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THE BOTANICAL REVIEW

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THE CHARACEAE, 1951[1]

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The Characeae is a family of algae of world-wide distribution and common occurrence. The group includes some 292 recognized species divided into six or seven genera, namely, *Chara, Nitella, Tolypella, Lychnothamnus, Lamprothamnium, Nitellopsis* and possibly the recently described *Protochara.* It is considered by a number of students (e.g., Migula, 1890; Pascher, 1925; Groves, J. and Bullock-Webster, 1920; Zaneveld, 1940) as comprising an assemblage of organisms sufficiently unique to merit the status of a separate division, the Charophyta. Smith (1938, 1950) includes it among the Chlorophyta as a class, Charophyceae, coordinate with the other green algae which comprise the class Chlorophyceae. Fritsch (1935) treated it as an order, Charales, giving his reasons for this disposition, and later (1944) rephrased even more clearly his basis of opinion for not considering the group a distinct division. The Characeae do exhibit a few characteristics not found among other green algae, but the majority of the characteristics would indicate alignment with the greens, as suggested by the latter two writers.

Members of the Characeae frequently present the appearance of a feathery equisetum when seen growing in several feet of water. Their relatively large size, as well as general appearance, might easily mislead the casual observer to consider them as higher plants, such as *Myriophyllum* or *Utricularia.* The species of this group display a wide range of variation in superficial aspects, such as size of thallus, relative size of different vegetative structures, and habit of growth. The mature plants range in size from tiny species two or three centimeters high to robust examples five or six

[1] Supported in part by the Research Fund of the College of Arts and Sciences of the University of Rhode Island.

feet long; and from forms with compact heads to those loose and flexible in make-up. The basic morphological characteristics underlying these variations are much more uniform than might be suspected, and there is a common structural pattern throughout the group. Marked morphological differences are few, and these have become accepted as genus and tribe characters. It is an assemblage of algae which is unique and closely knit, yet it displays no apparent close relationship to other known cryptogams.

From an evolutionary point of view, the existence of such a closely knit group of organisms, relatively isolated morphologically from other forms, at once raises interesting problems of their lines of descent and stimulates interpretations that can be placed on their existing peculiarities. Although not yet conclusive, the concurrence of a number of structural peculiarities tends to indicate that there seems to be little evidence against and more evidence for assuming an origin in or a mutual origin with the Chaetophorales. Among these peculiarities are a few that might be noted, such as the alternate nodes and internodes (cf. *Draparnaldiopsis*), cortication, and in particular certain aspects of the sex organs which have been discussed to a certain extent by Goebel (1930) and to a greater extent by Wood (unpublished paper, AAAS, 1947). A report of this paper is in preparation.

Interest in these organisms has been largely academic, since their economic value is neither great nor spectacular. Zaneveld (1940) summarized nine types of uses for which these plants have more or less economic importance, namely, fish culture, water purification, food for aquatic animals as well as farm stock, fertilizer, polishes, mud baths, therapeutic value, sugar clarification and insect control. A few notes with respect to certain of these possible uses may be worth adding to Zaneveld's earlier analysis. Concerning their value as water-clarifying agents and use in wildlife management, the report by Crocker (1948) of the plight of North Bay and Back Bay, Virginia, is particularly interesting. The damage to these valuable hunting and fishing areas as a result of removing locks and widening channels into the bay was critical. Aquatic seed plants were practically eliminated, and waterfowl left the region followed by the sportsmen. The Boyce Thompson Institution, after considered study, recommended replacing the locks. This recommendation involved the prediction that *Chara* and *Nitella*

would first cover the bottom and stabilize the shifting silt with resulting clarification of the water, and then seed plants would repopulate the region. The results followed the prediction, and within three years Characeae almost completely carpeted the bays and the turbid water became crystal clear. A later report indicated that the ducks and sportsmen had returned in gratifying numbers. The value of Characeae directly as food plants for waterfowl is well known. McAtee (1939) listed them among the ten most valuable waterfowl food plants. References and statistics are cited by Zaneveld (1940). W. P. Baldwin of the Fish and Wildlife Service has informed the present writer of observations made at the Savannah Wildlife Refuge, S. C. In one 135-acre pool, 100 acres of which were vegetated by a nearly pure stand of *Nitella acuminata,* 2000 ducks (including 1500 pintails) were supported during November, 1950. During January, 1951, although the numbers had declined, 500 pintails and shovellers were observed feeding in the pool. The possibility of Characeae being implicated in epidemics of botulism of wildfowl has been investigated, and Quortrup and Sudheimer (1942) confirmed earlier reports by the finding of *Clostridium botulinum* in decaying matter, including *Chara.* The active toxin accumulated in such debris. Use of Characeae has occasionally been advocated for indoor aquaria, and Gordon (1943) cited their importance not directly as food but in supporting abundant growths of epiphytes and small animal herbivores which in turn provide food for aquarium fish. *Nitella axillaris* is commonly available from tropical fish dealers under the name *Nitella gracilis.* Growths of these organisms in the field may become so dense as to be a nuisance, as in the reservoirs of the Madras water supply (Rafay, 1933). Copper salts have been found effective (Cook, 1926) as killing agents. The danger of accumulation of the toxic metallic ion in the bottom silt precludes recommendation of repeated use of this measure in cases where the natural biota is to be maintained. More suitable measures are being reported continuously in circulars on pond management. The question of the larvicidal properties of Characeae is still not decided. The discovery of this apparent phenomenon by Caballero (1919), in which mosquito larvae are reported to fail to occur or pupate in water supporting Characeae, stimulated a number of investigations. The results in the laboratory and in the field

were conflicting, and little continued interest in further exploring the possibilities has been taken since 1940. It appears that if a larvicidal property does actually exist, it is not unique to Characeae, and is rather a resultant of the conditions commonly typical of habitat in which these aquatics grow.

Although the Characeae are hardly to be considered of great economic value, their usefulness as experimental material is well appreciated. They are doubtlessly the most available freshwater autophyte with macroscopic cells sufficiently large to compete in usefulness with the marine *Halicystis* and *Valonia*. It is possible that the existence of a number of different species of various habitat preferences from alkaline and mildly acid freshwater to brackish water (15 ‰ NaCl) has not been generally appreciated. It is likely that large cells with a natural salt tolerance between fresh and salt water would be more adaptable to experiments and apparatus of certain types than would either freshwater or marine plant material. The large size of the internodal axial cells, which approach four or five inches in length in some species, such as *Nitella praelonga* and *Chara ceratophylla*, but average closer to one or two inches in forms more commonly used in the United States, such as *Nitella flexilis, N. clavata* and *Chara Braunii*, renders these plants admirable for numerous physiological investigations. Among these are such problems as permeability of cytoplasmic membranes (e.g., Brooks, 1927; Hoagland and Broyer, 1942), ion accumulation (e.g., Irwin, 1926; Hoagland and Davies, 1929; Hoagland and Broyer, 1942), bioelectric and action potentials (e.g., Blinks and Pickett, 1940), ionic mobilities (e.g., Osterhout, 1939) and internal hydrostatic pressures (e.g., Harvey, 1942). The rapid cyclosis or protoplasmic streaming, which is relatively insensitive to shock, makes these cells valuable for studies of the properties of protoplasm, such as the interrelationships between viscosity and temperature (e.g., Umrath, 1934), streaming and action potentials (e.g., Hill, 1941), light and streaming (e.g., Josing, 1901), and streaming and hydrostatic pressure (e.g., Harvey, 1942).

The wide applicability of Characeae to experimentation creates the need for culture methods, in many cases pure culture methods. As yet, the writer is unaware of any success that has been achieved in developing a satisfactory pure culture technique for these organ-

isms. Growing plants can frequently be maintained in household aquaria where exposure to light is restricted to a few hours daily. Blinks has maintained an excellent stock of *Nitella clavata* for years in an outdoor watering trough at Pacific Grove, California, by occasional addition of nutrients. The writer has been informed that in Osterhout's laboratories, internodal cells are collected and maintained in pans of nutrient solution for several months if necessary prior to experimentation. These isolated internodal cells remain healthy and proliferate slightly, but apparently do not form new plants.

The ecological aspects of the Characeae have not been investigated very extensively. Stroede (1931, 1933) and Olsen (1944) have summarized a number of field studies concerning the tolerance of certain European species. Wood (in press) has begun preliminary work with the Woods Hole, Mass., species. The latter writer (1950) has pointed out that, although the Characeae are commonly considered pioneer plants of relatively short duration in a given habitat, in certain localities they have persisted for over 50 years. The abundance of lime-incrustation by Characeae is widely appreciated as a factor in marl formation and filling in of aquatic habitats, but it should be noted that relatively few of the species regularly accumulate such carbonate incrustations in sufficient amounts to produce a significant deposition. Among those species that do are *Chara vulgaris, C. contraria* and *C. hispida.* The species of *Nitella,* for the most part, accumulate little lime. Recent interest in classifying aquatic habitats along phyto-sociological lines has resulted in a number of categories involving various Characeae as dominant plants (e.g., Kornaś and Medwecka-Kornaś, 1948).

The widespread range of interest which involves Characeae more or less directly in research or applied fields frequently requires a knowledge of the species under study. Workers engaged in such projects can well look to the taxonomist to provide identifications and means of identification. The taxonomist's work is a necessary tool for such studies, and as such the taxonomist provides an essential service in the botanical world. Identification of these organisms has generally been considered to be rather difficult, and part of the difficulty seems to be attributable to the lack of a comprehensive treatment. In lieu of such treatment, the writer has

attempted to prepare two lists which may provide an interim aid to workers until such time as some student is able to complete the long-needed study. The first is a list of recognized species of Characeae of the world, and the second is a list of publications helpful in identifying Characeae from different regions and countries of the world.

The first list represents an attempt to assemble the species names of Characeae for the world which are recognized at the present time. The list must be considered tentative for several reasons. First, since no consistent monographic treatment of the world's species exists, the opinions of various writers differ sufficiently so that clear recognition or disposal of certain species has not as yet been achieved. Second, no critical re-investigations have been made as yet in the case of a number of species, especially those recently described from remote parts of the world; and as a result a certain number of those listed are likely to be assigned in time to synonymy. Third, although the writer has endeavored to seek out and record the findings of all taxonomic literature on Characeae, there still exists that uncertain reservoir of publications which as yet have not come to his attention. Concerning the makeup of the list, it will be noted that author citations include only reference to the sources of original description. The literature is cited only for those authors for whom the year and pages are indicated. The authority names have been written out in full to facilitate tracing the history of names, as in combinations, as well as a convenience in reference to the literature. It will be noted in cases of combinations by the same author who described a species earlier as a subspecific entity, that the author's name is included in parentheses. This, again, is done to help establish the origin and history of an existing combination. In accordance with reports (Rickett, 1951) of decisions reached at the Botanical Congress, Stockholm, 1951, " apud " has been dropped. The term " in " is employed both in the prior application (cf. Art. 48, International Rules) and in the place of " apud ". The term " ex " has been retained in its general usage.

In three cases where the generally employed names are later homonyms, the present writer has created new names. This has been done in an effort to develop a workable and systematic nomenclature. It is recognized that monographic study may very

likely result in the assigning of these new names to synonymy, but in the meantime a legitimate name is available for these species. A study of synonymy has required certain modifications in the usual use of certain names. No defense is given to support these usages, and it is hoped that such a defense will be included in a larger manuscript now in preparation.

The writer wishes to acknowledge the invaluable criticisms of G. O. Allen of England. He hastens to add that Allen is not to be held responsible for opinions that are herein contained, since he was not able to inspect the final copy of the manuscript.

TENTATIVE LIST OF RECOGNIZED SPECIES OF CHARACEAE

1. *Chara abnormiformis* Vilhelm, 1928[?]: rep. p. 16
2. *C. aculeolata* Kützing *ex* Reichenbach, 1832: 843 [1]
   syn.: *C. polyacantha, fide* Groves & B.-W., 1924: 47
3. *C. altaica* Braun, 1882: 148 [1853, ined.]
4. *C. amplexa* Groves & Stephens, 1933: 283
5. *C. arcuatofolia* Vilhelm, 1928[?]: rep. p. 16
6. *C. aspera* Detharding *ex* Willdenow, 1809: 298
7. *C. australis* R. Brown, 1810: 346
8. *C. baltica* (Hartman) Fries *ex* Aspegren, 1823: 65
9. *C. Baueri* Braun, 1847: 13
   syn.: *C. scoparia* Bauer *in* Reich. (*non* Chevallier)
10. *C. bohemica* (Vilhelm) Vilhelm, 1923a: 159
11. *C. brachypus* Braun, 1849b: 298 [synonymy questionable]
12. *C. Braunii* Gmelin, 1826: 646
    syn.: *C. coronata* Ziz. *ex* Braun

?13. *C. Brionica* Stapf [descr. not yet located; cf. Flor. Exsicc. Austro-Hung., No. 1585]

14. *C. Brittonii* T. F. Allen *ex* Robinson, 1906: 295
15. *C. Buckellii* G. O. Allen, 1951: 150 [Filarszky, 1936: 476, as *C. Buckelii, nom. nud.*]

?16. *C. burmanica* Pal, 1932: 83 (= ?*C. fibrosa, fide* Zaneveld, 1940: 153)

17. *C. canescens* Desvaux *in* Loiseleur-Deslongchamps, 1810: 139
    syn.: *C. crinita*

[1] The problem of the taxonomy of *C. aculeolata* and *C. polyacantha* can be resolved only by use of the type method. Pending such investigation, the writer follows Groves & Bullock-Webster.

18. *C. capensis* Meyer *ex* Kützing, 1857: 28
    C. ceratophylla Wallroth, 1815: 192 (= *C. tomentosa, fide* Groves & B.-W., 1924a: 36
19. *C. chrysospora* J. Groves & Stephens, 1926: 155
20. *C. compacta* Robinson, 1906: 296
21. *C. concinna* Durieu & Cosson *in* Durieu, 1859: 183
    syn.: *C. Duriaei* Braun
22. *C. conimbrigensis* Gonçalves da Cunha, 1943b: 142
23. *C. connivens* Salzmann *ex* Braun, 1835: 73
    C. conniventi-fragilis Hy, 1913: 41 (hybrid, *C. connivens* × *fragilis*)
24. *C. contraria* Braun *ex* Kützing, 1845: 258
25. *C. corallina* Klein *ex* Willdenow, 1805: 86
26. *C. corfuensis* J. Groves *ex* Filarszky, 1936: 477
    C. coronata Ziz. *ex* Braun, 1834: 353 (= *C. Braunii*)
27. *C. coronatiformis* Robinson, 1906: 272
    C. crassicaulis Schleicher, 1821 [*nom. nud.*] (cf. *C. vulgaris*)
    C. crinita Wallroth, 1815: 190 (= *C. canescens*)
28. *C. crinitiformis* Robinson, 1906: 260
    syn.: *C. excelsa, pro parte*
29. *C. cubensis* T. F. Allen, 1894a: 163
    C. Curtissii T. F. Allen *ex* Robinson, 1906: 272 (= *C. fibrosa, fide* Zaneveld, 1940: 163)
30. *C. delicatula* Agardh, 1824: 130 (*non* Desv., 1810) [*nom. illeg.*][2]
31. *C. denudata* Braun, 1847: 5
    syn.: *C. dissoluta*
32. *C. desmacantha* J. Groves & Bullock-Webster, 1920: 94
    C. dichopitys Braun, 1882: 121 (= *C. Ecklonii*)

*33.

34. *C. disjuncta* (Nordstedt) Trabut *ex* Feldmann, 1946: 82
    C. dissoluta Braun *ex* Leonhardi, 1864: 182 (= *C. denudata, fide* H. & J. Groves, 1895: 290)

[2] The problem of the taxonomy of *C. delicatula*, and the presumed synonyms including *C. verrucosa* Itzigsohn, 1850, and *C. capillacea*, should be solved by the type method.

* Deleted from galley proof and succeeding entry numbers are correspondingly to be corrected. This correction has been considered in computing the total number of species.

35. *C. Domini* Vilhelm, 1928[?]: rep. p. 13
36. *C. Drummondii* Braun, 1849a: 201
    syn.: *C. psilopitys*
    C. Duriaei (Braun *in* Durieu) Braun, 1867: 926 (= *C. concinna*)
    syn.: *C. concinna* [synonymy questionable]
37. *C. Ecklonii* Braun *ex* Kützing, 1857: 19
    syn.: *C. dichopitys*
    C. elegans (Braun *ex* T. F. Allen) Robinson, 1906: 283 (= *C. zeylanica, fide* H. & J. Groves, 1911: 41)
38. *C. erythrogyna* Griffith, 1849: 278
    syn.: *C. Thwaitesii*
39. *C. evoluta* T. F. Allen [1880: exsicc. No. 16, descr?], 1882: 41
40. *C. excelsa* T. F. Allen [1880: exsicc. No. 15, descr?], 1882: 43
    syn.: *C. crinitiformis, pro parte, fide* Robinson, 1906: 260
    C. fertilissima (Braun) Robinson, 1906: 286 (= *C. zeylanica, fide* H. & J. Groves, 1911: 41)
41. *C. fibrosa* Agardh *ex* Bruzelius, 1824: 21
    syn: *C. flaccida*
42. *C. filicaulis* Robinson, 1906: 285
43. *C. filiformis* Hertzsch, 1855: 81
    syn.: *C. jubata*
44. *C. Fischeri* Migula, 1904: 538
    C. flaccida Braun, 1849b: 296 (= *C. fibrosa, fide* Zaneveld, 1940: 162)
    C. foetida Braun, 1834: 354 (= *C. vulgaris*)
45. *C. formosa* Robinson, 1906: 296
46. *C. fragifera* Durieu de Maisonneuve, 1859: 185
    C. fragilis Desvaux *in* Loiseleur-Deslongchamps, 1810: 137 (= *C. globularis, fide* G. O. Allen, 1938: 50)
47. *C. fulgens* Filarszky, 1934b: 720
?48. *C. galioides* DeCandolle, 1813: 93
49. *C. globata* Migula, 1904: 537
50. *C. globularis* Thuillier, 1799: 472
    syn.: *C. fragilis*
51. *C. gobiana* Vilhelm, 1928[?]: rep. p. 15

52. *C. Grovesii* Pal, 1932: 85
    C. guatemalensis (Nordstedt) Robinson, 1906: 286 (= *C. zeylanica, fide* Zaneveld, 1941: 144)
    C. gymnophylla Braun, 1834: 354 (= *C. vulgaris*) [Feldmann, 1946: 94, prefers *C. squamosa*]
?53. *C. gymnopitys* Braun, 1852: 708 (= *C. fibrosa, fide* Zaneveld, 1940: 158)
    C. gymnopus Braun, 1867: 942 [earlier refs., *nom. nud.*] (= *C. zeylanica*)
    C. haitensis Turpin, 1817: pl. at p. 164 (= *C. zeylanica*)
54. *C. Handae* Pal, 1932: 86
55. *C. Hansenii* (Sonder) Ophel, 1947: 322
    syn.: *Lamprothamnus Hansenii* Sonder
56. *C. Hatei* Dixit, 1940: 235
57. *C. hereroensis* Nordstedt, 1900: rep. p. 2
58. *C. hirsuta* T. F. Allen, 1900: 301
59. *C. hispida* Linnaeus, 1753: 1156 [*pro parte*]
    syn.: *C. major, fide* Hy, 1913: 37
    C. Hookeri Braun, 1849a: 202 (= *C. Ecklonii*)
60. *C. Hornemannii* Wallman, 1853: 288 [as *C. Hornemanni*]
    C. horrida Wahlstedt, 1862: 24 (*non vidi*) (= *C. hispida, fide* H. & J. Groves, 1880: 132)
61. *C. hungarica* Filarszky, 1930: 655
62. *C. hydropitys* Reichenbach, 1834: 1669
63. *C. hypnoides* Robinson, 1906: 263
64. *C. imperfecta* Braun, 1867: 900 [possibly an earlier descr.]
65. *C. inconnexa* T. F. Allen, 1882: 40
    C. inconstans Braun *ex* Kützing, 1857: 28 (= *C. zeylanica*)
66. *C. inermis* Zaneveld, 1940: 198
?67. *C. infirma* Braun, 1882: 177 (*spec. dub., fide* Nordstedt, 1882: 22)
    C. intermedia Braun *ex* Brébisson, 1859: 151 (*nom. illeg., non* Lange, 1856–9: 703) (= *C. papillosa* Kützing, *fide* Groves & B.-W., 1924: 42 [cf. footnote to *C. aculeolata*]
68. *C. intumescens* Robinson, 1906: 268
69. *C. javanica* Braun, 1849b: 300 (as *spec. dub.*)
    C. jubata Braun *ex* Kützing, 1857: 19 (= *C. filiformis*)
70. *C. Kenoyeri* Howe, 1929: 159

71. *C. keukensis* (T. F. Allen) Robinson, 1906: 273
72. *C. Kieneri* Daily, 1949: 127
?*C. Kokeilii* Braun, 1847b: 27 (= *C. vulgaris*)
C. Krausii Braun *ex* Kützing, 1857: 22 (= *C. phaeochiton, pro parte; C. Kraussiana, pro parte; fide* J. Groves & Stephens, 1933: 281)
73. *C. Kraussiana* J. Groves & Stephens, 1933: 285
syn.: *C. Krausii, pro parte*
?74. *C. Lamyana* Soulat-Ribette, 1892: 35
C. latifolia Willdenow, 1809: 298 (= *C. tomentosa*)
75. *C. leiopitys* Whelden, 1946. 306 [Whelden has informed the writer that "*leiopytis*" in the original text was a typographical error]
76. *C. leptopitys* Braun, 1882: 119
77. *C. leptosperma* Braun, 1882: 184 [1853, ined., 1860, *nom. nud.*]
C. Liebmanni Robinson, 1906: 271 (= *C. hydropitys, fide* H. & J. Groves, 1911: 39)
C. Liljebladii Wallman, 1853: 314 (= *C. baltica, fide* H. & J. Groves, 1881: 354)
78. *C. longifolia* Robinson, 1906: 272
C. Macounii (T. F. Allen) Robinson, 1906: 281 (= *C. aspera, fide* Zaneveld, 1940: 188)
C. macropogon Braun, 1843: 116 (= *Lamprothamnium macropogon* (Braun) Ophel)
C. major Vaillant *ex* Hy, 1913: 37 (= *C. hispida*)
C. Martiana Braun *ex* Nordstedt, 1882: 186 (*non* Wallman) (= *C. Rusbyana*)
C. Martiana Braun *ex* Wallman, 1853: 294 (= *C. sejuncta*)
79. *C. mauretanica* G. Feldmann, 1945: 171
C. mexicana (T. F. Allen) Robinson, 1906: 274 (= *C. hydropitys, fide* H. & J. Groves, 1911: 39)
80. *C. mollusca* Braun, 1882: 116
81. *C. Morongii* Robinson, 1906: 270
82. *C. muscosa* J. Groves & Bullock-Webster, 1924b: 33
83. *C. myriophylla* F. Müller *ex* Braun, 1852: 707
?84. *C. Nordhoffiae* (T. F. Allen) Robinson, 1906: 277
85. *C. nuda* Pal, 1932: 81
86. *C. oedophylla* Feldmann, 1945: 168

87. *C. pallida* Robinson, 1906: 258
88. **C. Palmeri** Wood, nom. nov.
    syn.: *Chara stellata* Robinson, 1906: 288 (*non* Gray, 1821: 28, = *N. tenuissima, fide* H. & J. Groves, 1880: 164)
89. *C. papillosa* Kützing, 1834: 707
    syn.: *C. intermedia* Braun (*non* Lange) [cf. footnote to *C. aculeolata*]
90. *C. Pashanii* Dixit, 1935: 258
91. *C. pelosiana* Avetta, 1898: 235
92. *C. phaeochiton* (Braun) Braun, 1882: 180
    syn.: *C. Krausii, pro parte*
93. *C. pistianensis* Vilhelm, 1923b: rep. p. 2
    C. plebeja R. Brown *ex* Braun, 1843a: 118 (= *C. australis, fide* Braun, 1882: 107)
    C. polyacantha Braun *ex* Leonhardi, 1864: 199 (= *C. aculeolata, fide* Groves & B.-W., 1924a: 48 [cf. footnote to *C. aculeolata*]
    C. polyphylla Braun, 1835: 70 (= *C. zeylanica*)
94. *C. poopoensis* G. O. Allen, 1940: 155
    C. Preissii Braun, 1843: 118 (= *C. Ecklonii*)
95. *C. pseudo-brachypus* J. Groves & Stephens, 1926: 156
96. *C. pseudogymnophylla* Filarszky, 1926: 8
    C. psilopitys Braun *ex* Nordstedt, 1882: 19 (= *C. Drummondii*)
    C. Robbinsii Halsted, 1879: 183 (= *C. hydropitys, fide* H. & J. Groves, 1911: 39)
    C. Rohlenae Vilhelm, 1912: 30 (= *C. vulgaris*)
    C. rudis (Braun *in* Wahlstedt) Braun *ex* Leonhardi, 1864: 185 (= *C. hispida, fide* H. & J. Groves, 1880: 132)
97. *C. Rusbyana* Howe, 1929: 160
    syn.: *C. Martiana* Braun *ex* Nordstedt
98. *C. salina* Schur, 1870: 202
99. *C. Sancta-Margaritae* (T. F. Allen) Robinson, 1906: 289
100. *C. scepusiensis* Filarszky, 1926: 8, 12
101. *C. Schaffneri* (Braun) T. F. Allen, 1888: 58
    syn.: *C. contraria* ssp. *Schaffneri*
    C. Schneckii Robinson, 1906: 271 (= *C. hydropitys, fide* Zaneveld, 1940: 170)

102. *C. Schroederi* Migula, 1914: 183 (*non vidi*)
C. Schweinitzii (Braun) Kützing, 1857: 31 (= *C. Braunii*)
C. scoparia Chevallier, 1827: 127 (*non* Bauer *in* Reichenbach, 1829: 1596 [cf. *C. Baueri*])
103. *C. sejuncta* Braun, 1845: 264 (ft. note)
104. *C. sibirica* Migula, 1904: 537 (= ?*C. hirsuta*)
105. *C. socotrensis* Nordstedt *in* Kuhn, 1883: 241
C. squamosa Desfontaines, 1800: 331 (= *C. vulgaris, fide* Zaneveld, 1940: 184) [Feldmann, 1946: 94, considers this distinct from *C. vulgaris*]
106. *C. stachymorpha* Ganterer, 1847: 19
C. stellata Robinson, 1906: 288 (*non* Gray, 1821: 28, = *N. tenuissima, fide* H. & J. Groves, 1880: 164) (= **C. Palmeri,** nom. nov.)
107. *C. strigosa* Braun, 1847: 16
108. *C. submollusca* Nordstedt, 1888a: 189
109. *C. succincta* Braun *in* Ascherson, 1878: 257
110. *C. tanyglochis* H. & J. Groves, 1906: 286
C. tehuacanensis Samano Bishop, 1932: 233 (= ?*C. contraria*) [Amelia Samano Bishop, in a letter to this writer, holds no further responsibility for this species. No types were preserved]
111. *C. tenera* Hasslow, 1939: 817
112. *C. tenuispina* Braun, 1835: 68
C. Thwaitesii Braun, 1882: 132 [1859, ined.] (= *C. erythrogyna, fide* G. O. Allen, 1927: 337)
113. *C. tomentosa* Linnaeus, 1753: 1156
syn.: *C. ceratophylla; C. latifolia*
C. trichacantha (Braun) Robinson, 1906: 294 (= *C. zeylanica, fide* H. & J. Groves, 1911: 41)
C. variabilis Filarszky, 1934b: 721 (= *C. zeylanica, fide* Zaneveld, 1940: 210)
C. verrucosa Itzigsohn, 1850: 338 [*nom. provis.;* hence, *nom. illeg.*] (= *C. delicatula* [*nom. illeg.*]) (cf. *C. delicatula*)
?114. *C. verticulata* Hate, 1909: 762
115. *C. vulgaris* Vaillant *ex* Linnaeus, 1753: 1156
syn.: *C. foetida*
116. *C. Wallichii* Braun, 1882: 107 [1854 ined.; other early refs. apparently *nom. nud.*]

117. *C. zeylanica* Klein *in* [?] Willdenow, 1805: 184 [generally dated 1803, but this has not been confirmed by the present writer]

Characias Rafinesque, 1815: 209 (= *Chara, fide* Wood, 1948a: 282)

Charina verticillata Filarszky & G. O. Allen *ex* Filarszky, 1936: 490 (= *Nitella* sp., *fide* Groves)

Charopsis Kützing, 1843: 319 (= *Chara, pro parte;* = *Lychnothamnus, pro parte*)

1. *Lamprothamnium aragonensis* (Prósper, 1910: 91) Wood, nov. comb.

   Basonym: *Lamprothamnus aragonensis* Prósper

2. *L. macropogon* (Braun) Ophel, 1947: 322

   syn.: *Chara macropogon, Lychnothamnus macropogon, Macropogon australicum*

3. *L. papulosum* (Wallroth) J. Groves, 1916: 336

   syn.: *Lamprothamnus; Lychnothamnus alopecuroides*

4. *L. toletanus* (Prósper, 1910: 93) Wood, nov. comb.

   Basonym: *Lamprothamnus toletanus* Prósper

Lamprothamnus aragonensis Prósper, 1910: 91 (cf. *Lamprothamnium aragonensis,* nov. comb.)

L. Hansenii Sonder, 1890: 25 (= *Chara Hansenii, fide* Ophel, 1947: 322)

L. toletanus Prospér, 1910: 93 (cf. *Lamprothamnium toletanus,* nov. comb.

Lychnothamnus alopecuroides (Braun) Braun, 1867: 798 (= *Lamprothamnium papulosum, fide* J. Groves, 1916: 337)

1. *Lychnothamnus barbatus* (Meyen) Leonhardi, 1863a: 57

L. macropogon (Braun) Braun, 1882: 103 (= *Lamprothamnium macropogon, fide* Ophel, 1947: 321)

L. stelliger (Bauer *in* Reichenbach) Braun, 1882: 102 (= *Nitellopsis obtusa, fide* J. Groves, 1919: 127)

Macropogon australicum Migula, 1891: 273 (= *Lamprothamnium macropogon, fide* Ophel, 1947: 321)

1. *Nitella abyssinica* Braun, 1882: 71
2. *N. acuminata* Braun *ex* Wallman, 1853: 263

N. africana (Braun) Kützing, 1849: 515 (= *Nitella mucronata, fide* Braun, 1867: 885)

3. *N. Alleninda* Zaneveld, 1940: 105
4. *N. Annandalei* Pal, 1932: 70
5. *N. annularis* T. F. Allen, 1894: 16
?6. *N. anomala* Wallman, 1853: 225
7. *N. Arechavaletae* Spegazzini, 1883: 224
   N. arvenica Hy, 1889: 429 (= *N. gracilis, fide* Hy, 1913: 14)
8. *N. Asagrayana* Schaffner *ex* Nordstedt, 1882: 67
   N. australis (R. Brown) Kützing, 1857: 11 (= *Chara australis, fide* Braun, 1882: 105)
9. *N. axillaris* Braun, 1858: 356
10. *N. axillocarpa* J. Groves & Stephens, 1933: 275
11. *N. Baronii* H. & J. Groves *in* Baker, 1887: 537 [as *N. Baroni*]
12. *N. Bastinii* T. F. Allen, 1894d: 10 [as *N. Bastini*]
    N. batrachosperma (Reichenbach) Braun, 1847: 10 (*non C. batrachosperma* Thuillier, *haud* Reichenbach) (= *N. confervacea, fide* G. O. Allen, 1950: 33)
13. *N. bengalensis* Kundu, 1941: 844
14. *N. bipartita* Filarszky, 1934b: 706
15. *N. Blankinshipii* T. F. Allen, 1892b: 5
16. *N. Blowiana* J. Groves, 1928: 133
17. *N. bonaërensis* Spegazzini, 1883: 224
    N. borealis Wallman *ex* Zetterstedt, 1839: 620 (= *N. gracilis, fide* Nyman, 1884: 878)
?18. *N. brachyteles* Braun *ex* Leonhardi, 1864: 173 (= ?*N. translucens, fide* Zaneveld, 1940: 70)
    N. Brebissoni (Hy) Hy, 1913: 8 (= *N. confervacea, fide* Corillion, 1946: 89)
19. *N. burmanica* Pal, 1932: 76
20. *N. californica* T. F. Allen, 1894b: 166
21. *N. capillaris* (Krocker) J. Groves & Bullock-Webster, 1920: 96
    syn.: *N. capitata*
22. *N. capillata* Braun, 1867: 893
    N. capitata (Nees von Esenbeck) Agardh, 1824: 125 (= *N. capillaris, fide* Groves & B.-W., 1920: 96)
23. *N. capitellata* Braun, 1882: 56
?24. *N. capitulifera* T. F. Allen, 1896c: 534 (= ?*N. acuminata, fide* Zaneveld, 1940: 57)

25. *N. cernua* Braun, 1858: 354
26. *N. clavata* Kützing, 1849: 518 (Bertero *ex* Braun, 1849a: 195, *nom. nud.*)
27. *N. Comptonii* J. Groves, 1922a: 69
28. *N. confervacea* (Brébisson) Braun *ex* Leonhardi, 1863b: 146
    syn.: *N. batrachosperma, N. Nordstedtiana*
29. *N. conformis* Nordstedt, 1880: 18
30. *N. congesta* (R. Brown) Braun, 1849a: 198
    N. conglobata Braun, 1882: 75 [1859, ined., 1867: 797, *nom. nud.*] (= *N. Lhotzkyi*)
31. *N. conifera* J. Groves & Stephens, 1933: 273
32. *N. coreana* Morioka, 1941a: 67
33. *N. cristata* Braun, 1852: 706
34. *N. dictyosperma* H. & J. Groves, 1898: 324
35. *N. diffusa* Braun, 1882: 80
36. *N. dilatata* T. F. Allen 1894d: 13
37. *N. dimorpha* Migula, 1930: 213
    N. dispersa Braun, 1867: 797 (and 1882: 47) (= *N. flagelliformis, fide* Zaneveld, 1940: 66)
38. *N. divaricata* J. Groves & Stephens, 1926: 149
39. *N. Dixonii* J. Groves, 1915: 41
40. *N. Doidgeae* J. Groves & Stephens, 1933: 279
41. *N. Dregeana* Braun *ex* Kützing, 1849: 517
    syn.: *N. tricuspis*
42. *N. dualis* Nordstedt, 1889: 7
43. *N. Duthieae* J. Groves & Stephens, 1933: 277
44. *N. elegans* Pal, 1932: 73
45. *N. expansa* T. F. Allen, 1898: 76
46. *N. fallosa* Morioka, 1940: 293
47. *N. fascicularis* Filarszky & G. O. Allen *ex* Filarszky, 1936: 478
48. *N. flagellifera* J. Groves & G. O. Allen, 1927: 337
49. *N. flagelliformis* Braun, 1849b: 294
50. *N. flexilis* (Linnaeus, *pro parte*) Agardh, 1824: 124
    N. formosa T. F. Allen, 1893a: 119 (= *N. hyalina, fide* T. F. Allen, 1893b: 258)
51. *N. furcata* (Roxburgh *ex* Bruzelius) Agardh, 1824: 124
    syn.: *N. polyglochin* Braun, *sens. strict., fide* J. Groves & G. O. Allen, 1927: 338

52. *N. gelatinosa* Braun, 1849a: 198
N. Glaziovii Zeller *in* Warming, 1876: 428 (= *N. microcarpa, fide* Nordstedt, 1882: 72)
53. *N. globulifera* Pal, 1932: 69
54. *N. gloeostachys* Braun, 1849a: 196
N. glomerulifera (Braun) T. F. Allen, 1889: 356 [*nom. illeg., non* Kützing, 1857: 32, = *T. glomerata*] (= *N. acuminata, fide* Wood, 1948: 359)
55. *N. gracilens* Morioka, 1941a: 64
56. *N. graciliformis* J. Groves, 1928: 128
57. *N. gracilis* (Smith) Agardh, 1824: 125
58. *N. gracillima* T. F. Allen, 1898: 76
N. grandis Braun *ex* Kützing, 1857: 14 (= ?*N. Dregeana*)
59. *N. guineensis* Kützing, 1849: 515
N. Gunnii Braun *ex* Nordstedt, 1882: 10 [synopsis] (= *N. penicillata* Braun, *fide* Braun, 1852: 704)
60. *N. habrocoma* J. Groves & Stephens, 1933: 278
N. havaiensis Nordstedt, 1878: 24 (= *N. trichotoma, fide* Nordstedt, 1882: 15, 88, 91)
61. *N. heterodactyla* Filarszky & G. O. Allen *ex* Filarszky, 1936: 480
?62. *N. heterophylla* (Braun) Braun, 1849a: 198 (*spec. dub., fide* Nordstedt, 1882: 14)
63. *N. heteroteles* J. Groves & Stephens, 1933: 277
64. *N. Hookeri* Braun, 1849a: 199 (*non* Reinsch, 1876)
65. *N. hyalina* (DeCandolle) Agardh, 1824: 126
66. *N. inaequalis* J. Groves, 1928: 127
67. *N. intermedia* Nordstedt *in* T. F. Allen, 1880: exsicc. No. 2
N. interrupta Braun *ex* Leonhardi, 1863b: 146 (*non* Kützing, 1849: 516, = *N. hyalina, fide* Braun, 1867: 890) (= **N. Leonhardii**, nom. nov.)
68. *N. japonica* T. F. Allen, 1893a: 120
69. *N. Knightiae* J. Groves & Stephens, 1933: 273
N. Lamyana Hy, 1889: 429 (= *N. gracilis, fide* Hy, 1913: 14)
70. *N. laxa* T. F. Allen, 1896b: 533
71. *N. Lechleri* Braun *ex* Horn af Rantzien, 1950a: 205 [3]

[3] Published as a *nov. comb.*, *N. Lechleri* (Braun) Horn af Rantzien, but this usage is illegitimate. The basonym, *N. trichotoma* ssp. *Lechleri* Braun

72. *N. Leibergii* T. F. Allen, 1896d: 19 (as *N. Leibergi*)
73. **N. Leonhardii,** Wood, nom. nov.
    syn.: *N. interrupta* Braun *ex* Leonhardi, 1863b: 146 (*non N. interrupta* (Ruprecht) Kützing, 1849: 516 (= *N. hyalina, fide* Braun, 1867: 890))
?74. *N. leptoclada* (Braun) Braun, 1882: 50 (= *N. sphaerocephala, pro parte, fide* J. Groves, 1928: 130)
75. *N. leptodactyla* J. Groves, 1922: 99
76. *N. leptosoma* Nordstedt, 1880: 17
77. *N. leptostachys* Braun, 1849: 199
78. *N. Lhotzkyi* (Braun) Braun, 1849: 197
    syn.: *N. conglobata*
    N. Lindheimeri Braun, 1858: 355 (= ?*N. acuminata*)
?79. *N. longifurca* (Ruprecht) Kützing, 1849: 515 (= ?*N. mucronata*)
80. *N. Macounii* (T. F. Allen) T. F. Allen, 1888: 11
81. *N. madagascariensis* Zaneveld, 1939: 373
?82. *N. mauritiana* Braun, 1867: 889 (= *N. acuminata, fide* Braun, 1882: 37; but most recent writers retain this species, cf. Zaneveld, 1940: 57)
    N. maxceana T. F. Allen, 1896d: 27 (= *N. Morongii, fide* Wood, 1949: 16)
83. *N. megacarpa* T. F. Allen, 1880: exsicc. No. 3
    syn.: ?*N. microcarpa*
84. *N. mexicana* T. F. Allen, 1894a: 165
85. *N. microcarpa* Braun, 1858: 357
    N. microglochin Braun *in* Zeller, 1873: 193 (= *N. microcarpa, fide* Zaneveld, 1940: 103)
86. *N. microphylla* Braun, 1849a: 196
87. *N. minuta* T. F. Allen, 1888: exsicc. No. 34
88. *N. mirabilis* Nordstedt *ex* J. Groves, 1924: 364
?89. *N. missouriensis* T. F. Allen, 1892: 5
90. *N. moniliformis* Zaneveld, 1940: 79 (*non* Morioka, 1941: 61; cf. *N. Moriokae, nom. nov.*)
91. *N. monodactyla* Braun, 1882: 28

(1882: 88), was illegitimate when published because it was superfluous through prior existence of *N. Zeyheri* Braun ex Kützing, 1857: 15. Braun included the latter species in creating *N. trichotoma.* Horn af Rantzien's usage constitutes, in fact, a new name attributed to Braun. That author, in correspondence with the writer, has agreed with this interpretation.

92. *N. montana* T. F. Allen, 1892b: 4
93. **N. Moriokae** Wood, nom. nov.
    syn.: *N. moniliformis* Morioka, 1941: 6 (*non* Zaneveld, 1940: 79). Named in honor of Mme. Hideo Morioka.
94. *N. Morongii* T. F. Allen, 1887: 214
    N. mucosa (Nordstedt) J. Groves, 1922b: 100 (= *N. pseudoflabellata, fide* Zaneveld, 1940: 75)
95. *N. mucronata* (Braun) Miquel, 1840: 428
96. *N. multipartita* T. F. Allen, 1898: 80
97. *N. musasiensis* Morioka, 1941a: 65
98. *N. muthnatae* T. F. Allen, 1887: 211
99. *N. myriotricha* Braun *ex* Kützing, 1857: 15
    N. Nordstedtiana H. & J. Groves, 1890: 66 (= *N. conferva-cea, fide* G. O. Allen, 1950: 33)
    syn.: *N. batrachosperma*
100. *N. obtusa* T. F. Allen, 1892a: 230
101. *N. occidentalis* T. F. Allen, 1894a: 166
102. *N. ogivalis* J. Groves & Stephens, 1933: 276
103. *N. oligogyra* Morioka, 1941a: 60
104. *N. oligospira* Braun, 1858: 357
105. *N. opaca* (Agardh *ex* Bruzelius) Agardh, 1824: 124
106. *N. orientalis* T. F. Allen, 1894b: 524
?107. *N. ornithopoda* Braun *in* Leonhardi, 1863b: 146 (= ?*N. gracilis, fide* Hy, 1913: 14, *pro parte*)
108. *N. partita* Nordstedt, 1891: pl. 1 (cf. also, 1918)
109. *N. patula* J. Groves & G. O. Allen, 1927: 338
110. *N. paucicostata* T. F. Allen, 1894b: 525
111. *N. penicillata* Braun, 1849: 196
    syn.: *N. Gunnii, fide* Braun, 1882: 46
112. *N. phauloteles* J. Groves, 1927: 262
113. *N. plumosa* Braun, 1867: 891 [1843, *nom. nud.*]
114. *N. polycarpa* Pal, 1932: 77
115. *N. polycephala* (Braun) Kützing, 1857: 13
    N. polyglochin Braun *ex* Nordstedt, 1889: 7, *sens. lat.* (= *N. microcarpa, pro parte, fide* Zaneveld, 1940: 103; Groves, 1928: 132)
116. *N. polygyra* Braun *ex* Kützing, 1857: 17
117. *N. praeclara* J. Groves & Stephens, 1926: 148

118. *N. praelonga* Braun, 1882: 40
119. *N. pseudoflabellata* Braun *in* Nordstedt, 1880: 16
N. pseudograciliformis Filarszky, 1934b: 707 (= *N. mucronata, fide* Zaneveld, 1940: 98)
120. *N. pseudotenuissima* Kundu, 1941: 843
121. *N. pulchella* T. F. Allen, 1895: 69
122. *N. pygmaea* Braun, 1882: 65
123. *N. remota* Braun, 1882: 45
124. *N. rigida* T. F. Allen, 1898: 73
125. *N. Robertsonii* Braun, 1882: 46 [1859, ined. as *N. Robertsoni*]
N. Roxburghii Braun, 1849b: 293 (= *N. furcata, fide* Zaneveld, 1940: 92)
126. *N. Saitoiana* T. F. Allen, 1898: 74
127. *N. Sonderi* Braun, 1852: 704
128. *N. spanioclema* J. Groves & Bullock-Webster, 1919: 1
129. *N. sphaerocephala* J. Groves, 1928: 130
syn.: *N. leptoclada, pro parte*
130. *N. spiciformis* Morioka, 1941a: 63
131. *N. stabilis* Imahori, 1949: 3
?132. *N. stellaris* T. F. Allen, 1896c: 534 (= ?*N. acuminata, fide* Zaneveld, 1940: 57)
N. stelligera (Bauer *in* Reichenbach) Kützing, 1843: 318 (= *Nitellopsis obtusa, fide* J. Groves, 1919: 127)
133. *N. struthioptila* J. Groves & Stephens, 1933: 279
N. Stuartiana Müller *ex* Kützing, 1857: 11 (= *C. australis, fide* Holtz, 1906: 38)
134. *N. Stuartii* Braun, 1852: 704 (as *N. Stuarti*)
N. subglomerata Braun, 1858: 356 (= *N. acuminata, fide* Braun, 1882: 36)
?135. *N. sublucens* T. F. Allen, 1895: 70 (= ?*N. translucens, fide* Zaneveld, 1940: 70)
?136. *N. subspicata* T. F. Allen, 1896a: 6 (= ?*N. acuminata, fide* Zaneveld, 1940: 57)
137. *N. subtilissima* Braun, 1849a: 196
138. *N. sumatrana* Filarszky, 1934b: 709
139. *N. superba* Pal, 1932: 67
140. *N. syncarpa* (Thuillier) Chevallier, 1827: 125
141. *N. tanakiana* T. F. Allen, 1898: 74

142. *N. tasmanica,* F. Müller *ex* Braun, 1852: 707
143. *N. tenuissima* (Desvaux) Kützing, 1843: 319
?144. *N. transilis* T. F. Allen, 1896d: 24 (= ?*N. tenuissima*)
145. *N. translucens* (Persoon) Agardh, 1824: 124
146. *N. tricellularis* (Nordstedt) Nordstedt *in* T. F. Allen, 1888: exsicc. 40a
N. trichotoma Braun, 1882: 88 (*nom. illeg.*) (cf. *N. Zeyheri, N. Lechleri*)
N. tricuspis Braun, 1867: 877 (= *N. Dregeana, fide* H. & J. Groves, 1906: 287)
147. *N. tuberculata* Kundu, 1937: 223
148. *N. tumida* Nordstedt, 1891: pl. 4 (cf. also, 1918)
149. *N. tumulosa* Zaneveld, 1940: 86
150. *N. uruguayensis* Horn af Rantzien, 1950a: 208
151. *N. vermiculata* J. Groves, 1928: 131
152. *N. Wattii* J. Groves, 1924: 367
153. *N. Zeyheri* Braun *ex* Kützing, 1857: 15
syn.: *N. trichotoma*

?1. *Nitellopsis aculeolata* Soulat-Ribette, 1892: 27
Nitellopsis inflata Filarszky & G. O. Allen *ex* Filarszky, 1936: 488 (= *Protochara inflata, fide* Womersley & Ophel, 1947: 314)
2. *Nitellopsis obtusa* (Desvaux *in* Loiseleur-Deslongchamps) J. Groves, 1919: 127
3. *Nitellopsis sarcularis* Zaneveld, 1940: 114
Nitellopsis stelliger (Bauer *in* Reichenbach) Hy, 1890: 46 (= *Nitellopsis obtusa, fide* J. Groves, 1919: 127)

1. *Protochara australis* Womersley & Ophel, 1947: 316
2. *Protochara inflata* (Filarszky & G. O. Allen *ex* Filarszky) Womersley & Ophel, 1947: 314
syn.: *Nitellopsis inflata*

1. *Tolypella apiculata* Braun, 1882: 98 [1838, 1853, ined.]
2. *T. californica* Braun, 1882: 99
3. *T. comosa* T. F. Allen, 1883: 112
4. *T. fimbriata* T. F. Allen, 1883: 113
5. *T. giennensis* Prósper, 1910: 83
6. *T. glomerata* (Desvaux *in* Loiseleur-Deslongchamps, *pro parte*) Leonhardi, 1863b: 129
7. *T. hispanica* Nordstedt *ex* T. F. Allen, 1888c: 51

8. *T. intertexta* T. F. Allen, 1883: 115
9. *T. intricata* (Trentepohl *ex* Roth) Leonhardi, 1863a: 57
10. *T. longicoma* Braun, 1882: 96 [1855, ined.]
11. *T. nidifica* (O. Müller) Leonhardi, 1863a: 57
    T. Normaniana (Nordstedt *in* Norman) Nordstedt, 1882: 95 (= ?*T. nidifica, fide* J. Groves & B.-W., 1920: 141)
12. *T. prolifera* (Ziz[iani?] *ex* Braun) Leonhardi, 1863a: 57
    T. Stenhammariana (Wallman) Soulat-Ribette, 1892: 38 (= *T. nidifica, fide* Braun, 1882: 93)
13. *T. stipitata* T. F. Allen, 1883: 114
    Tolypellopsis Migula, 1891: 253 (= *Nitellopsis* Hy, 1889: 398)
    Tolypellopsis simplicissima Filarszky, 1934b: 716 (= *C. australis, fide* Zaneveld, 1940: 129)

Number of species and genera

Genera	Number of species
1. *Chara*	116
2. *Lamprothamnium*	4
3. *Lychnothamnus*	1
4. *Nitella*	153
5. *Nitellopsis*	3
6. *Protochara*	2
7. *Tolypella*	13
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As no single work treats all the Characeae of the world, students attempting identification are dependent upon a number of different publications. Below, the writer, again with the valuable counsel of G. O. Allen, has attempted to bring together a list of the works which seem to be most valuable to him. The works useful over wide regions are entered first in a geographically arranged list. The works of particular value for specific localities or countries are entered in the major portion of the list which is arranged alphabetically by countries. The references indicated are to be found in the literature cited at the end of this review.

GUIDE TO SOME USEFUL TAXONOMIC LITERATURE FOR CHARACEAE

I. World:

Braun & Nordstedt, 1882

II. Regions:

Europe: Migula, 1890–1897; Groves, J., and Bullock-Webster, 1920, 1924a; Sydow, 1882
Africa: Braun, 1867; Groves, J., and Stephens, 1927, 1933; Feldmann, 1946
Asia: Zaneveld, 1940; Pal, 1932
Oceania: Zaneveld, 1940
Australia: Nordstedt, 1918; Groves, J., and G. O. Allen, 1935
North America: Robinson, 1906; Allen, T. F., 1883; Wood, 1948c
South America: Horn af Rantzien, 1950b

III. Countries or localities:

Alaska: Saunders, 1901; Hasslow, 1934
Albania: Filarszky, 1893, 1926a
Algeria: Feldmann, 1945, 1946
Annam: Groves, J., 1921
Arabia: [Braun, 1882]
Argentina: Horn af Rantzien, 1950b; Spegazzini, 1883
Australia: Nordstedt, 1891, 1918 [cf. also Queensland]
Austria (Germany): Leonhardi, 1864
Bahamas (W. Indies): Hitchcock, 1898; Britton & Millspaugh, 1920
Belgium: Crepin, 1863 [cf. also Netherlands]
Bohemia (Germany): Leonhardi, 1863a, b; Vilhelm, 1923a
Bolivia: Allen, G. O., 1940; Hasslow, 1940
Borneo: Groves, J., 1914; Zaneveld, 1940
Brazil: Hasslow, 1934
Bulgaria: Petkoff, 1914, 1922; Vilhelm, 1907, 1914
Burma: Pal, 1932
Canada: Allen, T. F., 1883; Robinson, 1906; Wood, 1948c
Cape Peninsula (S. Africa): Groves, H. & J., 1906; Groves, J., and Stephens, 1926, 1933
Ceylon (India): Groves, J., 1922b
Chili (S. America): Hasslow, 1934
China: Jao, 1947, 1947a

Colombia (S. America): Braun, 1858; Zaneveld, 1941
Cuba (W. Indies): Groves, H. & J., 1898; Zaneveld, 1940
Czechoslovakia: Vilhelm, 1923b
Danzig: Lakowitz, 1907, 1929
Denmark: Olsen, 1944
East Indies: Zaneveld, 1940
Ecuador (S. America): [Horn af Rantzien, 1950b]
Egypt: [Braun, 1882]
Fiji I. (Polynesia): Allen, T. F., 1887
Finland: Cedercreutz, 1932; Hirn, 1900, 1906
France: Hy, 1913, 1914; Corillion, 1946, 1947
Germany: Migula, 1890–1897
Great Britain: Groves, J., and Bullock-Webster, 1920, 1924a; Allen, G. O., 1950
Greece: [Migula, 1890–1897]
Guam I. (Polynesia): Groves, J., 1921
Guiana (S. America): Braun, 1858
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HISTOCHEMICAL LOCALIZATION OF ENZYMES IN VASCULAR PLANTS

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INTRODUCTION

The study of enzyme localization in cells and tissues is different in cadre and goal from the discipline of biochemistry and enzymology. Localization of enzymes offers a procedure for tissue characterization, and elucidation of enzymes in the primary developing tissues of the plant has topographical value in studies of differentiation. The formation of specific enzyme systems and their localization in ontogeny are parts of, if not primary forces in, the process of differentiation. There are several other objectives for histochemical localization of enzymes. Evidence may be found to classify tissues on the fundamental principle of homology. The basis of tissue function may be revealed, and observations may be made which aid in adducing the inherent integrity of form and function.

The conventional methods of enzymology do not afford evidence for localization of enzymes in specific tissues. In the standard homogenate methods of enzyme study, in which a score of funda-

mentally different tissues in the plant are ground and extracted, there is at best only a generalized depiction of enzyme distribution. The accepted methods of enzymology, while desirable in their own right, do not give information on the histological inception, distribution or function of enzyme systems.

It has been repeatedly emphasized (cf. Kertesz, 1937; Schneider, 1949) that there is a need for caution in drawing conclusions regarding the activity of enzymes and their localization in tissues where direct observation of reactions in the tissues is used. There are disadvantages in direct tissue methods where reactions are observed under the microscope, namely, localized absorption of indicators, the possibility that the tissue in which an indicator reaction is observed may not be the locus of activation, and numerous related artifacts that are inherent in all localization methods. In the improved indirect methods of Linderstrøm-Lang, Holter and Glick, many of these objections have been eliminated by the refinement of macro methods (cf. reviews by Glick, 1935, 1939, 1949*a*, 1949*b*; Linderstrøm-Lang, 1939; Linderstrøm-Lang and Holter, 1940, 1941).

Direct methods designed to reveal qualitatively the distribution of the functional enzyme systems, rather than the effects resulting from histolysis and gross extraction methods, are of manifest importance where enzyme systems being studied are dependent upon cellular integrity. Cytolysis may result in a loss of activity of certain enzymes, and Yudkin (1937) has presented evidence that enzymes appear to be linked with the structure of the cell, as was pointed out earlier by Quastel (1926) and by Penrose and Quastel (1931) in a study of dehydrogenase activity.

Enzyme reactions are controlled in living cells, but when tissues are sectioned, end-products and intermediates may appear or may be detected. Oxidase reaction is not a " vital reaction " but one of dying or dead cells, as pointed out by Staemmler (1927) and Combes (1928). Peroxidases, catalases, lipoxidases, phenolases, and esterases may become activated in sectioning and wounding to give characteristic abnormal end products: aldehydes, oxidized fats, free fatty acids, aglycones, tannins, etc. These enzyme systems, which are independent of cellular stability, may be readily detected and are consistently observed in certain tissues as pre- or post-mortem phenomena.

Dehydrogenase activity, however, may be inseparably associated with the integrity of the cell. Dehydrogenases are partially or completely destroyed by lysis or disturbance of the normal state of the tissue. Destruction by freezing or grinding of meristems impairs the dehydrogenase and hydrogen donor systems apparently responsible for the reduction of triphenyltetrazolium chloride to the red insoluble formazan (Roberts, 1950). Similar disturbances to meristems may, however, activate several of the oxidases, glucosidases, lipases, etc. The colorimetric methods used in detecting these post-mortem enzyme systems are not satisfactory from a quantitative viewpoint, but they are useful in a general qualitative way for delineating various tissues where the results are consistent and where control methods and sections are used for comparisons.

There are direct methods of observing enzyme localization where the substrate may be observed to undergo changes. Amylases and phosphorylases may be studied in specific tissues where direct changes in starch content may be observed. End-product accumulation associated with pathological conditions in localized areas has been used to postulate the general type of activator systems present (Dufrenoy, 1945, 1947). Direct methods of this type have been used so infrequently, however, as to suggest that histochemists and physiological anatomists do not consider them reliable or useful.

It is the intention of this review to survey the work that has been done on histochemical localization of enzymes in vascular plants, to discuss the relative merits of some of the methods used, and to present the contributions of this approach to problems in differentiation and maturation.

OXIDIZING ENZYMES: OXIDASES AND DEHYDROGENASES

Enzymes which activate the oxidation of phenols, fatty acids, sugars, amino acids, etc., probably by addition of oxygen, are categorized as "oxidases". Discussion of the localization of these enzymes will be presented according to the convenient classification of oxidases used by Sumner and Somers (1947): those containing iron (catalase, peroxidase, cytochrome oxidase); those containing copper (polyphenol oxidase, laccase, ascorbic acid oxidase); miscellaneous oxidases (lipoxidase and unclassified oxidases). Dehydrogenase enzymes, which activate the loss of hydrogen, resulting in oxidation, are also oxidizing enzymes, and the meager informa-

tion available on localization of dehydrogenases in plant tissues will be discussed in this section on oxidizing enzymes.

PEROXIDASE. Peroxidase catalyzes the oxidation of many chromogens, various phenols and aromatic amines in the presence of peroxides. Hydrogen peroxide is applied to sections in the most common test used, and organic peroxides (peroxidized turpentine, ether, unsaturated fatty acids, etc.) have also been used, but the results are not so clear as with hydrogen peroxide. The botanist Raciborski (1898) found a substance, oxidizing gum guaiac in the presence of hydrogen peroxide, which he designated "leptomin" because of its localization in the sieve tubes and companion cells of the phloem. A month later Linoissier isolated, in material of animal origin, a similar substance that he named "peroxidase". Peroxidase action was discovered earlier in 1863 by Schönbein.

Raciborski (1898, 1902, 1905) localized leptomin (peroxidase) in the phloem, lenticels, guard cells, gap cells of the endodermis and the "absorption zone" of roots. He found alpha naphthol and hydrogen peroxide to be better than guaiac or dimethyl paraphenylenediamine as indicators. The danger of oxidation of indicators by exposure to air and localized absorption of indicators by cell membranes were apparent to Raciborksi; it is interesting to note that he recognized the function of peroxidase as an oxygen carrier and compared its role in plants with haemoglobin and haemocyanin in animals. Grüss (1898) classified oxidases as α, β and γ on a basis of correlation between guaiac/hydrogen peroxide reactions and the distribution of starch; Raciborski (1898*b*) criticized this attempt to correlate diastatic action with peroxidase.

Wodziczko (1915, 1916) repeated the classic researches of Raciborski and noted a strong reaction of endodermal walls when benzidine and hydrogen peroxide were used to detect peroxidase; he expressed the opinion that this characteristic of the endodermis might be classified as an extracellular enzyme reaction. Richter (1939) found peroxidase reactions, using luminol as the indicator, in the same tissues listed by Raciborski.

Using qualitative indicators (benzidine, guaiac, alpha naphthol, para phenylenediamine, etc., plus hydrogen peroxide), the distribution of peroxidase has been repeatedly observed. Peroxidase activity has been reported for the epidermis and vascular bundles by

several workers (Atkins, 1916; Hussein and Cruess, 1940; Rosoff and Cruess, 1949). Citrus fruits contain peroxidase in the peel and albedo (Willimott and Wokes, 1926; Davis, 1942). Benzidine reactions are reported to reveal peroxidase in the aleurone (Palladin and Manskaja, 1923), and the aleurone, embryo and cotyledons of dry seed (Coupin, 1925). According to the review by Sullivan (1948), the distribution of peroxidase in wheat and other cereal grains has not been studied, but color tests indicate that the bran contains a greater quantity than portions of the grain. From similar indicator tests cytological observations are said to reveal that: peroxidases are localized in vacuoles and chondriosomes (Mangenot, 1928); intense peroxidase reaction may be observed in male spores of *Equisetum* (Joyet-Lavergne, 1927); the inner walls of oil cells are lined with material which shows peroxidase reaction (Yahagi, 1928); cytoplasm and nucleus give reactions for peroxidase (Palladin and Manskaja, 1923).

Brieger (1924) found strong reactions for peroxidase in wound zones, especially in the underlying living cells near the surface of the wound, and he stated that peroxidase is a characteristic early reaction associated with wounding.

A correlation is stated to exist between greening of the epidermis and peroxidase content (Rischow, 1927), but Lilienstern (1928) found the production of peroxidase to be in inverse proportion to the chlorophyll content in extract methods of study of peroxidase in *Cuscuta* and its host.

Many of these observations are perhaps useful in a general qualitative way for delineating tissues, but there are several objections that must be raised. There is absorption of oxidized quinones by cell walls; it has been repeatedly observed that xylem vessel walls not only oxidize these indicators but also absorb the oxidized products. The indicators for peroxidase are oxidized by iron and copper salts (Valdiquie, 1923; Dubois, 1923; Moog, Garrigue and Valdiquie, 1939). In a critical study of histochemical peroxidase reactions, Biniecke (1939) found that various metals give reactions for peroxidase with the commonly used indicators. Using several oxidizable chromogens in the presence of hydrogen peroxide and salts of copper and iron, it has been found that ascorbic acid acts as a peroxidase (Bezssonoff and Leroux, 1945). In a survey of tissues in the bean pod, thermostable benzidine/hydrogen peroxide

reactions were found, and the authors (Isaac and Winch, 1947) concluded that benzidine is not a reliable indicator.

It has been known for some time that aldehydes in plant tissues may give peroxidase reactions with benzidine, guaiac and other chromogens (Woker, 1914). Reeve, in an interesting and valuable paper (1949), has confirmed the fact that aldehyde and peroxidase reactions may be demonstrated with the same reagents. With acid, cyanide or heat treatment of solutions or sections, Reeve has developed a series of histochemical tests, in which differential reactions may be used to distinguish between peroxidases and aldehydes. With acid, heat (boiling for 2 to 3 minutes) or cyanide pre-treatment, the peroxidase reactions were much slower and gave distinctive colors with the indicator paraphenylenediamine. Additional work on the interpretation of aldehyde reactions, as outlined by Danielli (1949) and Cain (1949), should be of help in investigations of peroxidases. It is the opinion of the writer that the aldehyde indicator thiobarbituric acid, introduced by Bernheim, Bernheim and Wilbur (1948, 1950), could be used in topographic studies to differentiate aldehydes and peroxidases.

A method which eliminates the objectionable features of the indicator method (localized absorption of indicators, interference by free iron and copper, interference by aldehyde reactions) is found in the application of sodium thiosulphate to fresh sections to bring about formation of the pink hemochromogen of peroxidase. The reduced compound of peroxidase was first observed spectroscopically in horse-radish root tissue by Keilin and Mann (1936) when they established the porphyrin protein nature of the peroxidases. Van Fleet (1947) used a Zeiss microspectroscope to detect some of the characteristic absorption bands of peroxidase and found localization primarily in the phloem. With this method it was possible to verify the work of Raciborski on "leptomin". Using sodium fluoride, sodium thiosulphate and sodium hydrosulfite, various colored hemochromogens of peroxidase were formed, and it was observed that the heaviest localization of peroxidase was in the phloem. Peroxidase was also found to be localized in meristems, including wound meristems, the epidermis, endodermis and the entire phloem area, including sieve tubes, companion cells and surrounding parenchyma (Van Fleet, 1947). In an interesting investigation of legume nodules with the microspectroscopic ocular,

Smith (1949) detected the presence of haemoglobin and peroxidase in the tissues.

While the indicator plus hydrogen peroxide tests are on theoretical grounds not completely reliable because of other enzyme systems and free cellular heme oxidations, it is a fact that the tissue patterns obtained with indicators are similar to those obtained with spectroscopic method. It would appear that some of the objections to the use of indicators, raised by Biniecki and others, have been met by the hydrosulfite reduction of peroxidase and by use of the microspectroscopic ocular.

CATALASE. Catalase activates the decomposition of hydrogen peroxide to form water and molecular oxygen. This enzyme is general in its distribution in plant tissue and apparently common to all mature tissues. The earliest work on catalase topography was done on sugar-beet roots by Stanek (1908). Very few additional observations have been made on the histological distribution of catalase. Direct observation has been used in a non-specific way to show that catalase activity is associated with living tissue (Lakon, 1922), with non-chlorophyll-bearing tissues in variegated plants (Bustinza, 1930) and with plasmolized cell surfaces (Chodat, 1936).

Catalase has been correlated with the iron and chloroplast content of plant tissue in gross studies. Hydrogen peroxide in fresh 1% solution has been applied to sections and examined directly for bubble release; the bubble-free tissues are said to be low in catalase, but it is apparent that the release of oxygen from hydrogen peroxide may be more strongly displayed where intercellular spaces are present than in the compact tissues. The released oxygen may diffuse from cell to cell in the sections without becoming gaseous except in tissues with well-developed intercellular spaces. The basis of the observed reactions on pieces of tissue has not been demonstrated by any final proof to be due to catalase.

Helm (1931) applied hydrogen peroxide to meristem sections and found that the more compact procambial strands and meristem cells do not react and that the "meristem ring" could be detected because it did not react. Thus the position and arrangement of procambial tissues were detected, but the reaction was not demonstrated to be due to localization of catalase in surrounding parenchyma. In a similar method of observation (Lopriore, 1928; Zeis-

ler, 1938), the active stigmatic surface of pistils, in surface areas where pollen grains germinate and are able to penetrate, was shown to contain a very active catalase. The same workers found ripe pollen grains to be higher in catalase than younger pollen.

Oxygen release from hydrogen peroxide measured manometrically has been used to determine catalase activity in portions of various tissues. In apples the xylem, inner and outer bark, callus tissue, buds and skin tissue of fruit were high in catalase (Heinicke, 1924; Harding, 1936). Krossing (1940) separated spinach leaves into fractions and found the strongest catalase activity in the chloroplasts and cytoplasm; the water-soluble material did not have any catalase activity, but the cell-wall material contained weak catalase action.

CYTOCHROME OXIDASE. When a mixture of alpha naphthol and dimethyl-*p*-phenylenediamine in dilute sodium carbonate is oxidized by plant or animal tissue, a purple indophenol is formed. Equal volumes of dilute solutions are mixed just before application to the sectioned or whole tissue, and the nadi (naphthol and diamine) reaction is observed. The nadi reagent autoxidizes slowly and oxidizes readily in the presence of metals. The reaction must be rapid if one is to assume the enzyme is present.

The enzyme, formerly called indophenol oxidase, is now known as cytochrome oxidase because it has been found that the enzyme oxidizes cytochrome C which then oxidizes nadi reagent. The indophenol oxidase reaction has been reported for several plant tissues, but some of the early work, although consistent in its tissue delineation, is probably not reliable, even in a qualitative way, because of lack of theoretical knowledge concerning the nature of the reaction. Cytochrome oxidase determined with the nadi reagent has been reported to be localized in the epidermis of leaves, root hairs and the nuclear and cytoplasmic membranes of various cells (Czapek, 1902; Reed, 1915). Interpretation of results is complicated by the presence of reducing substances in some tissues which appear to induce a weaker nadi reaction (Czapek, 1902). Numerous observations that are difficult to interpret have been made on the pronounced reaction of nadi with xylem and other lignified elements; the difficulty lies in the failure to use control sections, lack of interest in applying previously oxidized nadi to study absorption, and failure to use known inhibitors. Many of the reactions

observed may be caused by aldehydes and the presence of laccase which oxidizes the reagent. The nadi oxidation may also result from peroxide oxidants in unsaturated lipids (Lison, 1935).

The role of cytochromes is now known in the action of indophenol oxidase. The cytochromes, first identified in plants by Keilin (1928), are believed to bring about the oxidation of nadi reagent (Stotz, Sidwell and Hogness, 1938). The indophenol oxidase in pollen oxidizes cytochrome C (Okunuki, 1939); the oxidase of tea leaves, in fermentation following damage, has been found to be cytochrome oxidase and is firmly bound to the tissue (Roberts, 1940, 1942).

Brown and Goddard (1941) point out that the classical color reactions used in detecting indophenol oxidase are not reliable; the reaction in the indophenol test is a result of oxidase (phaeohemin) and carrier (cytochrome) action, and the test does not distinguish the two components. Brown and Goddard found the alpha absorption bands of cytochrome A and C in extracts of wheat germ; and Goddard (1944) isolated cytochrome C from wheat embryos. Further work on portions of various separated tissues would be of interest in plotting the distribution of cytochrome and would obviate some of the objectionable features of the qualitative nadi test.

A method for the use of buffered nadi reagent on fresh tissue from frozen sections is outlined by Glick (1949). The reagent is mixed with a buffer in the pH range 3.5 to 5.5, which is said to be suitable for plant tissue.

POLYPHENOL OXIDASE. This copper-containing enzyme catalyzes the oxidation of various phenols in the presence of gaseous oxygen. The term " polyphenol oxidase " is inclusive and is frequently used to include tyrosinase, monophenol oxidase and catecholase. Polyphenol oxidase was first observed in vascular plants in 1896–98 by Bourquelot, Herissey and Bertrand (see Diener, 1926). They found the black coloration of the seed coat of *Vicia faba* to be related to tyrosine oxidation. Bertrand found that the red coloration of sugar-beet sap was associated with tyrosine oxidation, and that the tyrosinase (polyphenol oxidase) was localized in and around the vascular bundles. Following this classic work many observations were made on the localization of polyphenol oxidase; various indicators have been used (benzidine, catechol, guaiacol, gum gua-

iac, several aminophenols, pyrogallol, etc.) on fresh sections of stems and roots and particularly of fruits and tubers.

The polyphenol oxidase system has been found to be activated after wounding or general necrosis. Diener (1926) observed the oxidation and post-mortem blackening of the epidermis, ground tissue and the vascular bundle tissues of leaves of *Vicia faba* when a quinine substrate was used. According to Diener, the reaction was localized in cell walls; the nuclei and chloroplasts were observed to either react with or strongly absorb the oxidized products. Blackening is caused in these tissues also by autoxidation of dihydroxyphenylalanine (Chodat and Evard, 1928).

Crushing of potato tissue increased the phenol oxidase activity 15 times, according to Szent-Györgyi and Vietorisz (1931). Whether inhibiting factors were destroyed, activators were freed, or enzyme and substrate were in separate tissues was not known, but Szent-Györgyi found a substance, ascorbic acid, responsible for a lag in the oxidation of phenols. In the presence of horse-radish peroxidase, oxidation of ascorbic acid and reduction of quinones proceeded until all of the ascorbic acid was oxidized (Tauber, 1936). When the ascorbic acid was finally oxidized, the phenolic colored decomposition products appeared. Thus phenolic substances may not be oxidized in intact undisturbed tissues because of the presence of reducing substances and other antioxidants. Robinson and Nelson (1944) reported that darkening of potato tubers when exposed to air may be attributed to dislocation or disruption of reducing substances in the cells. Pre- or post-mortem activation of phenol oxidases, which are inactive in uninjured plant tissues, may be related to natural immunity in the accumulation of bactericidal quinoids, according to Szent-Györgyi and Vietorisz (1931).

Dufrenoy (1945, 1947) has suggested that production of red quinoidal substances may be related to respiratory disturbances and "decompensation respiration" in certain tissues (parenchymal, perivascular and vascular). Reed and Dufrenoy, (1942; also Dufrenoy, 1945; MacDougal and Dufrenoy, 1946) have stated that oxidizable penols and polyphenol oxidase are in the vacuolar sap where oxidation is prevented normally by hydrogen donors (ascorbic-dehydroascorbic acid, various sulfhydryl systems, etc.); with senescence, nutrient deficiency or injury, the oxidation-reduction equilibrium is disturbed and the polyphenol oxidase system is activated.

Localization of phenol oxidase in potato and artichoke tubers was surveyed by Belval et al. Belval and Laenffer (1946) found polyphenol oxidase in the potato tuber localized in the subero-phelloderm layers and cells in the vascular bundles. Using guaiac and nadi reagent (which would have been oxidized through the cytochrome oxidase system as well), it was observed that parenchymal cells were negative as compared with normal or wound periderm cells and vascular tissues. The polyphenol oxidase was present in all cells, but the complete phenol/polyphenol oxidase system, i.e., the substrate and enzyme, was localized, as evidenced by internal chromogen oxidation in the tissues named. The enzyme was present in all cells; the general oxidation of introduced indicators was observed to take place to some extent in all cells. This is in agreement with the findings of Belval and Legrand (1946*a*, 1946*b*) on the localization of phenol/polyphenol oxidase in artichoke tubers; the phenol/polyphenol oxidase system was localized in the phelloderm and vascular tissue, but polyphenol oxidase was general in its distribution.

A unique method of qualitative detection of phenolase in potatoes is described by Molisch (1923). Reagent paper soaked with tetramethylparaphenylenediamine, known as "Wurster's paper" or "tetrapaper", was placed between freshly cut halves of potato. The cork cambium gave a blue oxidation "print", and later the inner parenchyma reacted. Wurster's paper gave reactions for the epidermis and vascular ring in stems of *Helianthus annuus*.

Polyphenol oxidase activity in fruit tissue of apples is said to be localized in or near the epidermis and in the vicinity of the vascular tissue of the core, as indicated by oxidation with guaiac, benzidine and tetramethyl-*p*-phenylenediamine (Drain, 1923). Polyphenol oxidase activity is not apparent in apple tissue until all of the ascorbic acid is oxidized, according to Ponting and Joslyn (1948). Localization of the enzyme system may therefore be a reflection of the distribution of reducing substances and their oxidation. Polyphenol oxidase in apricots is also, as in apple fruits, localized in the epidermis and the bundle sheath cells (Samisch, 1935). Ascorbic acid oxidation and polyphenol oxidation apparently take place in the skin tissue of tomato fruits by disruption of the tissue which frees the oxidase (Wokes and Organ, 1943).

Indicators applied to etiolated potato stems appeared to react strongly with plastids, suggesting localization of polyphenol oxidase

(Chodat and Rouge, 1922); using more definitive methods, Li and Bonner (1947) found polyphenol oxidase in the grana of chloroplasts; the chlorophyll was extracted from plastids, obtained by the method of Granick (1938), and the oxidase was not affected by the extraction. Using similar methods, Arnon (1948) separated the cytoplasm from chloroplast fragments of chard leaves and found polyphenol oxidase to be localized in the chloroplasts when catechol and ascorbic acid were used as substrates (Arnon, 1948).

There are numerous observations in the literature on unclassified "oxidases" which appear, in the light of recent classification, to be polyphenol oxidases. Sections of proliferating leaves of *Bryophyllium* treated with guaiac revealed an increase of polyphenol oxidase in the parenchyma of the notches at the time of regeneration (Freeland, 1933). There is apparently a polyphenol oxidase in the bundle sheath of *Ilex* leaves (Sanglet, 1928); vascular tissues are repeatedly reported to give reactions suggesting polyphenol oxidase systems (Czapek, 1921). Phenols in wood rays and wood parenchyma cells, which give a thermolabile guaiac reaction, are probably oxidized by polyphenol oxidase (Bailey, 1910). Polyphenol oxidase has also been reported in pollen (Lidfors, 1899; see Euler et al., 1944). These and many similar observations should be repeated because of revised theories and classifications of enzymes; in particular, localization of reducing substances should be studied in conjunction with polyphenol oxidase distribution.

The activity of the phenol/polyphenol oxidase system has been studied in the cortex of stems, and it has been found that the rate of oxidation in situ may be studied with an electronic photometer attached to the ocular of the microscope (Van Fleet, 1949). The activity of the phenolase system in cortical cells was found to be related to the inherent differentiation pattern and the site exposure from which the plants were obtained. The rate of phenol oxidase action in extracts has been studied in a similar method in which the rate oxidation of a reduced indicator was determined photometrically by galvanometer deflections (Smith, Robinson and Stotz, 1949). This latter method would appear to be suitable in succinct studies for indirect tissue examinations.

Ascorbic oxidase, another of the copper enzymes closely related to polyphenol oxidase, has been recently studied by Newcomb (1951) who presents an extensive and valuable paper on the general distribution and cellular localization of this enzyme. By dif-

ferential centrifugation of homogenates of pith, meristem, callus and other tissues, Newcomb found that ascorbic oxidase was associated with cell wall material. This fact, coupled with a demonstration that auxin caused a sharp increase in ascorbic oxidase activity and a concomitant cell enlargement, suggests that this enzyme plays some specific role in the growth of the primary wall. This work is significant in that it presents a new experimental approach to problems in enzyme localization and problems in differentiation and growth.

DEHYDROGENASES. Oxidation of substances in plant tissues may take place by removal of hydrogen from the molecules; the theory of activation and transfer of hydrogen was developed by Wieland. Thunberg showed that enzymatic dehydrogenation is a function of living cells, and he developed a special method for studying dehydrogenases. Thunberg and others have studied the distribution of dehydrogenases in tissues of mammals; unfortunately localization of dehydrogenases has not been extensively surveyed in vascular plants. Furthermore, of the many dehydrogenases described in in vitro studies, few or none have been directly localized in the tissue. The evidence for dehydrogenases is general and not specific; their localization with indicator methods has been for dehydrogenation rather than for specific dehydrogenases.

The term " dehydrogenases " was introduced by Thunberg, and he showed that they may be demonstrated by reduction of methylene blue to the colorless leucobase in a method which bears his name (see review by Thunberg, 1930). Thunberg (1930, 1936) found a number of seeds that contain dehydrogenases; he investigated pollen and found dehydrogenases that activate malic acid, glycerophosphoric acid and certain alcohols. Using the Thunberg method of study, it has been observed that ground garlic tissue reduces methylene blue, indicating the presence of dehydrogenases (Sugihara and Cruess, 1945); several dehydrogenases (of triosephosphate, alcohol and glutamic acid) have been found in lily pollen (Okunuki, 1939; see Euler et al., 1944).

The methods of Thunberg were also used in an interesting investigation of cambium, phloem and xylem by Griffioen (1938). Cambium was scraped from the inside of the cortex of stems and the surface of the xylem (the cortex was first split away at the cambial line); the tissue was washed and placed in Thunberg tubes

with methylene blue as the indicator. The reduced dye solutions (reduction accomplished in five hours at 30° C) were oxidized with air or hydrogen peroxide, and the original color was obtained, thus establishing that reduction of the dye had taken place and not absorption of the dye by the tissue. Reduction by cortical parenchyma and xylem was weaker than by the cambium. Tissues killed with hot water and used as controls showed no reduction at all. In histological micro studies, using the reduction of methylene blue, cambium showed the strongest reduction of the indicator and xylem the weakest, with the phloem somewhat intermediate on a time basis. Control sections were used and reoxidation of the indicator was induced. There is the possibility of error in the localized absorption of the indicator in this work, but the observations are reproducible and are substantiated by use of another indicator method said to reveal dehydrogenations and dehydrogenase localization. The indicator, 2,3,5-triphenyltetrazolium chloride, has been used extensively in attempts to localize centers of dehydrogenation and to elucidate specific dehydrogenases histochemically.

Living tissues readily reduce aqueous solutions of sodium selenite to the red or pink selenium, and triphenyltetrazolium chloride is reduced by viable tissues to the red insoluble triphenyl formazan. These reagents have been utilized to test the viability of seeds (Eidmann, 1938; Lakon, 1942; Mattson et al., 1947; Porter et al., 1947; Cottrell, 1947). It has been suggested by several workers that triphenyltetrazolium chloride is reduced to the red formazan by dehydrogenase enzymes present in embryo or meristem tissue (Mattson et al., 1947; Jensen et al., 1951; Roberts, 1951). Mattson et al. found that reduction of triphenyltetrazolium chloride is associated with dehydrogenase and requires coenzymes I or II. Jensen et al. have investigated dehydrogenase enzyme systems in preparations from corn embryos and have found several that reduce the tetrazolium reagent; it is probable, according to the authors, that reduction of the reagent by viable embryos can take place if one or more dehydrogenase systems with favorable redox potentials are present.

Selenium salts are reduced to colored products also by meristems. Shreiner and Sullivan (1911*a*, 1911*b*) described reduction of sodium selenite by root primordia; it was their opinion that the reduction of sodium selenite to selenium was related to non-enzy-

matic processes and substances, the oxyorganic acids or unsaturated compounds, e.g., unsaturated fatty acids (see also Dufrenoy, 1929).

Dufrenoy and Pratt (1948) and Pratt and Dufrenoy (1948), in a study of the localization of the sites of reducing activity (oxidation centers) in stalks of sugar cane, found that the intercalary and bud meristems, sieve tubes and "synapts" between phloem elements would reduce the tetrazolium reagent and also sodium selenite. The sites of reduction coincided with the location of oil droplets in the cytoplasm.

Roberts (1950) surveyed the tissues in several vascular plants that reduce tetrazolium salts. Apical meristems, intercalary meristems and lateral meristems, including cambium (cf. Waugh, 1948), cork cambium and the inner and outer meristematic zones of the developing cortex, showed varying degrees of reduction of the reagent. The inner and outer cortical zones in the meristematic root tissue have not been reported previously as zones of reduction of indicators.

Heating and freezing sections prior to addition of tetrazolium destroys the reaction, but if "carbowax" 4000 is used as an embedding medium in cutting sections with the freezing microtome, the reduction of tetrazoles is not completely destroyed (McLane, 1951). Any disturbance of the overall integrity of the tissue results in a lowering of the rate and intensity of reduction; the dependence of dehydrogenase function on cellular integrity has been demonstrated in bacteria by Quastel (1926) and others.

It appears highly probable that dehydrogenase enzymes are responsible for the reduction of tetrazoles by plant tissues. In a survey of factors responsible for the reduction of tetrazoles by meristems, Roberts (1951) applied enzyme and general metabolic inhibitors, in particular, some that have specificity for sulfhydryl protein, to embryos of corn. These inhibition studies are regarded by Roberts, due to lack of specificity for the reaction, as indicating that no one dehydrogenase system is responsible. It appears probable from the work of Jensen et al. (1951) with extracts, and of Roberts (1951) on direct tissue reactions, that the general redox potential level, maintained by several systems, brings about the reduction of tetrazolium. Roberts found that the sulfhydryl protein tissue pattern was similar to the tetrazolium reduction pattern in

sections of embryos; the reagent and method used for determining the sulfhydryl pattern was developed by Bennett (1948).

LIPOXIDASE. The term " lipoxidase " was applied by André and Hou (1932) to an enzyme they discovered in soy bean which oxidizes unsaturated fatty acids in the presence of atmospheric oxygen. This enzyme has been called " carotene oxidase " because of the concomitant destruction of carotene when an unsaturated fat is peroxidized; for a time the enzyme was also called " unsaturated fat oxidase " (Tauber, 1940; Strain, 1941; Van Fleet, 1943*a*). Classification of lipoxidase has not been completed, but isolation of it in the crystalline state by Holman (1947) has made illucidation of its properties and activity possible (Holman, 1948).

Hummel and Mattill (1944) come to the conclusion that lipoxidase is an aerobic dehydrogenase instead of an oxidase; in contrast Balls and Kies (1944) have suggested that lipoxidase is not an aerobic dehydrogenase because it will not reduce methylene blue anaërobically. Mikhlin and Pshennova (1946) have suggested that the name " lipoxidase " is not suitable for an enzyme which does not transport oxygen, and that it would be better to designate it as a peroxidizing enzyme.

Several workers have stated, from similar observations, that lipoxidase not only is capable of oxidizing unsaturated fatty acids but also may induce oxidation of guaiac and other indicators (André and Hou, 1932). *p*-Phenylenediamine and dihydroxyphenylalanine react with fat peroxides to give characteristic blue oxidation pigments, according to Strain (1941). Süllman (1941, 1942) found that lipoxidase from *Soya* does not oxidize guaiacum tincture, but that oxidation takes place when hydrogen peroxide is added; he was of the opinion that the enzyme solution therefore contained peroxidase and that the oxidation of carotenoids, guaiacum and other indicators is secondary. Earlier workers were of the opinion that indopenol oxidase action is dependent on lipoids (Vernon, 1912) and that oxidation of nadi reagent and other oxidase indicators by lipids is not enzymatic but is a result of the presence of peroxides of fats and other organic peroxides (Gallagher, 1923; Gutstein, 1929; Lison, 1935).

Van Fleet (1942, 1943*a*) found that sodium selenite in dilute solutions applied to sections of stems and roots induced the oxidation of several redox and oxidase indicators in those tissues which were

shown to have an accumulation of unsaturated fatty acids. Anatomical features of the endodermis were shown to be associated with variations in the behavior of an unsaturated oxidation system (Van Fleet, 1942, 1943*b*, 1950). However, the unsaturated fat oxidation system of the endodermis and related cell types may not be a function of lipoxidase localization. Holman (1948) found a sharp decline in lipoxidase activity in early stages of germination of soy beans; and he has pointed out that, " Contrary to the general findings of Van Fleet, lipoxidase activity was very low at the seedling stage in soy beans. It may well be that the Van Fleet test for oxidase activity detects fatty acid oxidases other than lipoxidase. However, prolonged high activity of lipoxidase should not be necessary for prolonged oxidation of its substrates. . . . Once the reaction is initiated, the oxidation can proceed by a chain mechanism. It may thus be that lipoxidase has its function only in initiating the oxidation of the unsaturated fatty acids . . .".

The fatty acids of the cells of the parenchymal vascular boundary react immediately with a number of redox indicators (Van Fleet, 1942*b*, 1950). The leuco base of a number of indicators (methylene blue, dichlorophenolindophenol dyes, etc.) is rapidly oxidized by the cells of the endodermis. The localized and immediate oxidation of these indicators in sections may result from a reaction between the unsaturated fatty acid peroxides and the leuco base dyes in the presence of peroxidase. Dam and his students (Glavind et al., 1948; Hartman et al., 1948; Hartmann et al., 1948; Glavind et al., 1949) have demonstrated that peroxidized fats and fatty acids react in the presence of hemin to oxidize leuco dyes, benzidine, α-naphthol and nadi reagent.

The leuco dyes may thus be used for localization of unsaturated fat peroxidation in fat-containing tissues, but these dye tests do not necessarily reflect lipoxidase activity; a more specific test for lipoxidase must be developed. Deposition of suberin and other oxidation and condensation products of unsaturated fatty acids is the only criterion of localized fat oxidation. Localization of pro- and antioxidants that influence the autoxidation of unsaturated fats is one method of histochemical approach to the problem of fat behavior related to differentiation (Van Fleet, 1949*b*), but it will not give any direct evidence of the presence or function of lipoxidase.

OXIDASES ASSOCIATED WITH THE DISTRIBUTION OF ANTHOCYANIN. Several workers have emphasized, from similar observations, that pigment distribution in flowers coincides with peroxidase and "oxidase" localization. The epidermal peroxidase, determined with benzidine and hydrogen peroxide, has been observed to be clearly defined in anthocyanin-bearing flowers, whereas the same tissue in white flowers did not give the peroxidase reaction (Keeble and Armstrong, 1912; Atkins, 1913, 1916). According to Mirande (1922), anthocyanin is developed only in cells containing oxidase, and St. Jonesco (1921) was of the opinion that the appearance of red pigments in plant tissues is due to oxidation of the precursor. Nagai (1921), however, studying the same problem, found that the oxidase from soy beans oxidizes anthocyanin to yellow or brown substances, and he concluded that oxidase enzymes destroy anthocyanin but do not bring about its formation.

The mechanism for oxidation of indicators in tissues which may later become anthocyanic has not been experimentally demonstrated. It is probable that anthocyanin, even in the dilution of early stages of pigmentation, may oxidize the common redox indicators. The oxonium form of anthocyanin may be a strong hydrogen acceptor. The oxonium salts of anthocyanin are probably present under the alkaline conditions imposed by the buffers used with nadi and other reagents employed by the early workers. Reichel (1937) found that anthocyanin could function as hydrogen acceptors, being reduced to catechin-like products, in Thunberg experiments when a purified aldehydrase dehydrogenated propylaldehyde.

In a survey of oxidation of *p*-phenylenediamine, α-naphthol, leuco methylene blue and related indicators in pigmented, with genetically comparable non-pigmented tissue for controls, it has been found that there is a localized absorption of the oxidized form of the indicators in the pigmented tissue (Van Fleet, 1945). Macro and micro iodimetric methods applied to this absorptive system revealed a localized iodine absorption in pigmented tissue. The iodine number of pigmented tissues was always higher than for comparable non-pigmented tissues. The higher iodine absorption of anthocyanin-bearing epidermal cells was demonstrated to be localized in cytoplasmic lipids, in lipoclastic bodies in the cell and

in lipids impregnating the cell walls. It appears that the oxidase system, believed by early workers to be causal in anthocyanin development, is in reality a reflection of the accumulation of unsaturated lipids which absorb and react with redox indicators. Anthocyanin thus may act to localize and increase the mean unsaturation of dermal tissues in which the pigment is localized.

CARBOHYDRASES

β-GLUCOSIDASE. This enzyme is found in almonds and other seeds of the Rosaceae, and it has been localized in stem and root tissues of several plants. It hydrolyzes the β-glucosides (amygdalin, salicin, prunasin, arbutin, etc.). The inclusive term "emulsin" was applied for a long time to preparations from almonds that hydrolyze the cyanogenic glucoside amygdalin and other glucosides. In the early work on the localization of "emulsin" (β-glucosidase), specific histochemical tests were not used. The localization tests were based on protein reactions. The enzyme-bearing cells gave characteristic reactions with copper sulfate, potassium hydroxide, orcin hydrochloride and general protein tests in which the biuret and Millon's reagent color tests were used (Johannsen, 1887; Guignard, 1890). The general protein tests used were not specific for any one enzyme.

Most of the early tests used for localizing β-glucosidase employed some method of detecting cyanide release from the glucoside amygdalin. The hydrocyanic acid released in amygdalin hydrolysis by the enzyme was commonly detected with picric acid. Guignard's delicate and difficult qualitative test using picric acid indicated a localization of amygdalin hydrolysis in the pericycle and bundle sheath; the yellow color of alkaline picramate was reduced to a red picramic acid easily observed in the tissues. This reaction was not highly specific for hydrocyanic acid; sugars at the alkaline level attained could bring about the reduction.

Tauböch (1936) applied a solution of amygdalin in chloroform in the presence of silver nitrate and observed localization of reduced silver. This method also leaves much to be desired but was an improvement over the older methods. Tauböch confirmed the early observations on localization of glucosidase in the bundle sheath system. In all cases the principle localization of this enzyme was in the dermal tissues, the bundle sheath or the zone around the bundles. Enzyme and glucoside are not in the same cells, but they

are brought together when the tissues are damaged in making sections.

The presence of tannins, phlobaphenes and tannoid oxyaromatics in the form of aglycones (hydrolized glucosides) in dermal, endodermal and perivascular tissues may be demonstrated to be a result of localization of the β-glucosidase/glucoside system. Glucosides, introduced into partially evacuated leaf and stem sections, were hydrolized, and a deposit of the corresponding aglycone was found primarily in the tissues of the parenchymal/vascular boundary (Van Fleet, 1946). Amygdalin in chloroform was applied to the sections, and the release of hydrocyanic acid was determined with silver nitrate in dilute solution with very dilute methylene blue, with cupric acetate in benzidine and with the standard Guignard test using 1% picric acid in 10% sodium carbonate. Deposition of blue crystals of silver cyanide was not sharply localized, the perivascular tissues reacted strongly; but other tissues—cambium, phellogen and xylem parenchyma—reacted with silver nitrate as well as substances associated with chloroplasts in the cortex. The cupric acetate and the picric acid tests for cyanide were easy to obtain, and comparison of amygdalin-treated sections was made with control sections lacking amygdalin. In many cases, differences between amygdalin-treated sections and the controls were observed. When both amygdalin and β-glucosidase (obtained by the method of Bridel and Desmaret, 1928) were applied to sections, there was a general but irregular release of cyanide throughout all tissues. Glucosidase action appeared to be localized in the cambium, the endodermis, pericycle and phellogen, but the results were somewhat irregular. Glucosidase localization in the parenchymal/vascular boundary was easier to obtain in *Prunus Laurocerasus* than in other plants surveyed (Van Fleet, 1950).

Hydrolysis of glucosides to aglycones and oxidation of the aglycone have been observed by indirect methods to follow a gradient pattern in the cortex (Van Fleet, 1948). The hydrolysis of glucosides and the concomitant oxidation of aglycones in fresh sections were observed in several species of *Baptisia*. The glucoside baptisin was hydrolized to the aglycone baptigenin which oxidized to a black quinoidal substance.

By similar indirect methods localization of glucosides and of the hydrolizing enzymes was studied long ago in the hydrolysis of indican. Beijerinck (1900) found that "emulsin" of almonds de-

composed indican and that the blue oxidized indoxyl was localized in parenchyma cells surrounding the vascular bundles. The metabolic function of the glucosides as reserves and of the aglycones as oxygen acceptors (see Boiteau, 1947) in specific tissues can not be studied, however, until better localization tests are devised.

AMYLASES. Although starch in plant tissues occurs in easily observed form, there has been very little work on the localization of amylases. Localization of starch in plastids, and observations that starch transformation probably takes place in the plastids suggest that amylase localization may be correlated with the distribution of plastids. Amylase function and the "starch–sugar" balance are modified by light, water, hydrion concentration and the concentration of products, but starch increase or decrease in the plastids may be used as a rough index to the localization of amylases. But qualitative studies on amylase and starch localization have not been numerous, even though the starch grain and the plastid are easily observed.

Amylase is said to be associated with the plastids, but the function of amylase at any given moment appears to be a function of obscure conditions and equilibria within and around the plastids (Chodat and Rouge, 1922; Hanes, 1937). Maige (1927, 1928, 1932) found starch formation to be a result of a catalytic agent secreted by the plastid, but he claimed starch digestion was by amylase in the cytoplasm. Hydrolysis of starch grains in cells of cotyledons began where the grains were in contact with the cytoplasm, not next to the plastid, suggesting a cytoplasmic origin of amylase. Krossing (1940) and Nesgovorov (1941) found, in leaves separated into chloroplast, cytoplasm, water-soluble and cell wall fractions, that the amylases were in the chloroplast fraction.

The most extensive work on amylase localization has been done on cereal grains; early experiments showed the amylase to be localized in the aleurone layer at the periphery of the endosperm. Sections were cut from the fruit and endosperm of germinating seeds and covered with a layer of starch paste; after 24 hours the starch grains showed corrosion in the area of the aleurone (Haberlandt, 1890). Direct observation of starch grain corrosion in barley grains suggested that amylase was secreted by the epithelial layer of the scutellum (Mann and Harlan, 1915). Horning and Petrie (1927) observed that amylases were secreted from the scu-

tellum and assumed they were associated with mitochondria; there were mitochondria in areas where amylase action on starch was observed. Bernstein (1943), confirming observations made by Lampe (1931), pointed out that starch accumulated first in abgerminal and distal cells, last in the cells next to the scutellum in sections of developing corn endosperm. Thus by direct methods of observation the action of β-amylase and its localization in the endosperm have been observed.

Linderstrøm-Lang and Engel (1937), using the well known microtitration methods developed by Linderstrøm-Lang and Holter (1934), found amylase localized in the cells between the aleurone and the starch-containing cells of the endosperm. Accurate methods of sectioning were perfected to obtain the separate tissues, thus eliminating the objectionable features of crude tissue scraping and direct qualitative observation used by previous workers. The amylase concentration was determined by the quantity of maltose released and the number of aleurone or other cell types per section. The sections were extracted with phosphate buffer, and the active α-amylase and inactive β-amylase, activated with papain, were determined by the quantity of maltose released (see review by Linderstrøm-Lang and Holter, 1940).

Using similar methods, Engel and Bretschneider (1947) made a comparative investigation of the distribution of amylase and mitochondria; they take exception to the observations of Horning and Petrie (1927) that amylase is associated with mitochondria; no relation was apparent between the number of mitochondria and the amount of amylase calculated per unit of tissue. Engel (1947*a*) reported in a valuable paper that the bran and aleurone cells of resting grains did not contain amylase, the first starch-containing layer (the sub-aleurone) was high in amylase, the inner endosperm contained amylase, and the endosperm next to the embryo was high in amylase; the amylase in the embryo was localized in the scutellum.

Degradation of starch grains in the living cell begins in spots and is not uniform over the surface of the grain. Empty hulls of starch grains, the sac-like sheaths or coats of the former grains, may be seen to be perforated by holes, the probable points of initial hydrolysis (Hanes, 1937). The external membrane of the starch grain is different from the grain proper in physical and chemical

properties. The action of amylases in the living cell, on a basis of circumstantial evidence, is presumably conditioned by the nature of the hull of the grain. The starch grains of the "starch sheath" in stems are more resistant to ptyalin, diastase and mineral acid than the starch grains of other tissues (Van Fleet, 1943*b*). In the "starch sheath" of *Smilax* several grains may be surrounded by a common suberic membrane which may interfere with amylases.

Starch in the living cell may not be subject to amylase action, either as a consequence of the coating over the grains or because of inhibition of amylase by surface-active substances, such as sodium oleate and lecithin (Mühlbauer, 1931). Localization in the endodermis of starch resistant to amylases may be a result of the presence of unsaturated fatty acids in the cells and the covering of the grains by hulls resistant to hydrolysis. Fatty acids, particularly unsaturated fatty acids, are known to inhibit amylase (Velluz, 1927). Externally applied factors have been found to inhibit digestion of starch; indole acetic acid inhibited starch digestion in the endodermis as compared with the controls (Bausor, 1942). An amylase inhibitor is present in mature wheat kernels but is absent from young developing kernels; a study was made of starch degradation in sections held in buffered saliva (Kneen and Sandstedt, 1946). Histochemical examination of ripening banana fruit was said to reveal that tannin inhibits amylase (Sastri, 1934).

The cells of the parenchyma/vascular boundary contain starch, particularly in the "parenchyma sheath" of the leaf and petiole. Daily starch synthesis and hydrolysis have been observed in the sharply defined sheaths surrounding the vascular bundles in *Setaria* and other plants (Alexandrov, 1926). Rhoades and Carvalho (1944) found that the specialized plastids of the parenchyma sheath deposited starch during the day; at night the starch was hydrolized. It was their opinion that starch was deposited only when the rate of movement of sugar into the bundle sheath from the mesophyll was greater than the rate with which sugar passed from the sheath cells into the vascular tissue. The bundle sheath is thus unique in having amylases, as well as esterases (lipases) and phenolases, that are clearly defined and readily modified in their activation by light and dark (Van Fleet, 1950); it is a fact, however, that the evidence for these enzymes is circuitous and indirect.

Plant tissues in sterile culture do not grow readily on starch and therefore cannot be assayed for amylase localization, but Nickell and Brakke (1950) (see also Brakke and Nickell, 1951) have found that virus tumor tissue from *Rumex acetosa* is able to utilize starch as the sole source of carbon. An extracellular amylase was found to be present in the medium, and a sensitive iodimetric/spectrophotometric assay was used to determine micro quantities of enzyme production. This method may provide further information about the factors influencing the activation and localization of the amylases.

CYTASES AND RELATED CARBOHYDRASES. Protopectinase and pectinase, the enzymes that hydrolize and dissolve protopectin, pectin, pectates and pectic acid, are associated with loss of the pectic middle lamella and with maturation and mellowing of fruit. Autolysis of some aerenchymal and lysigenous tissues, development of the pollen chamber in gymnosperms, breakdown of cortical cells where branch roots arise, and similar phenomena are in part expressions of "cytase" activation in localized areas. Protopectinase, pectinase and pectase (an esterase), hemicellulases and other carbohydrases are probably present in maturing parenchymal tissues where cellular separation and middle lamella lysis have been observed.

Paton (1921) found pectinase in the pollen of all of the many species tested, and she emphasized the fact that pollen tubes make their way between the walls of adjacent cells (by hydrolysis of middle lamellae) instead of by penetrating the walls. We should therefore expect to find pectinase most frequently and not cellulose-digesting enzymes; this was demonstrated to be true. Pectinase, amylase, invertase and catalase were found in all species of pollen tested. Tests were made for 13 kinds of enzymes, but the carbohydrases were the only enzymes present in all species; proteolytic enzymes, lipases and other enzymes were demonstrated in some but not in all. Pistils were incubated with freshly ground pollen, and a second set of pistils was incubated with boiled pollen. It was found that styles with the active pollen softened, while those with boiled pollen remained firm and could not be teased apart easily. Digestion of the middle lamella by active pollen was also observed in stem sections. Thymol was used to retard the growth of bacteria, and evidence was presented that the pollen and not associated microörganisms produced the enzymes.

Collenchyma is found in the aerial portions of herbaceous dicotyledonous stems but not commonly below the ground-line. The cortex of the stem and leaf petiole as well as roots do not commonly differentiate collenchyma unless exposed to light (Van Fleet, 1946*b*). The distribution of the collenchyma may be assumed to be a result of factors which control the distribution of hemicellulose; the hemicellulose deposit in the collenchymal cell is the characteristic anatomical feature. Hemicellulase activation and the carbohydrate balance apparently prevent deposition of hemicellulose in subaerial portions of the cortex, but hemicellulase is apparently inhibited in aerial herbaceous tissues. When the below-ground cortex of squash stems is scraped and ground in sand, an extract is obtained which slowly hydrolyzes the collenchyma in aerial stem sections of the same plant. Hydrolysis by subaerial cortical extracts is slower than that obtained with commercial hemicellulase prepared from fungi. Control sections incubated in extracts from the outer cortex of the above-ground stem show slight or no hydrolysis; control sections held in water show no hydrolysis. These experiments (Van Fleet, 1951) do not prove causation for the distribution of collenchyma and the localized activation of hemicellulase, but they may form the cadre for further study of the collenchyma. Similar methods were used by Grüss (1910) in a study of the hydrolysis of hemicellulose walls by the cytase in wound gum from cherry.

Deposition of hemicellulose has not been studied in terms of hemicellulase activation and distribution, but it has been pointed out by Murneek (1929) that hemicellulose deposited in early stages of development may be hydrolyzed later, probably through enzyme activation. The interesting seasonal changes in the callus of phloem, demonstrated by Esau (1948) in a survey of dormant and reactivated phloem, may be related to seasonal enzyme synthesis and hydrolysis of the callose by carbohydrases.

ESTERASES

LIPASE. Since the early observations by Green (1891) on the localization of lipase in the aleurone tissue of the endosperm of *Ricinus communis*, there has been very little work of significance on lipase localization until the more recent successful efforts of Pett (1935), Glick (1934) and Engel (1947*b*). By the methods

of Linderstrøm-Lang et al., Pett dissected and examined the bran, endosperm, scutellum, cotyledons and radicles of germinating wheat grains and studied the lipase action of these portions on methylbutyrate. He found the scutellum to be high in lipase until germination when the enzyme decreased; emphasis in the study was on gross portions of the grain rather than on individual tissues. By appropriate sectioning methods which he developed earlier (see Engel and Bretschneider, 1947), Engel separated the aleurone from the subaleurone layers of the endosperm. He found the aleurone and embryo to be rich in esterase, as determined by methods developed by Glick (1934) in which an acidimetric micro titration was used for estimating the hydrolysis of methylbutyrate to free butyric acid.

In the Gomori (1945, 1949) method of localization of lipase in animal tissues, sections are incubated in water-soluble fatty acid esters in the presence of calcium salts. Insoluble calcium soaps are deposited at the sites of lipase and are transformed to the corresponding lead salts and blackened with hydrogen sulfide. Glick and Fischer (1946) attempted unsuccessfully to localize lipase in wheat grain by the Gomori method.

Lipase activation has been observed in the bundle sheath and the hypodermis. When leaves are held in the dark for 20 to 40 hours, the neutral fat glycerides are hydrolyzed and free fatty acids appear in the dermal and perivascular tissues. The evidence for this change from neutral fats to free fatty acids is based on the use of polychrome Nile blue or polychrome methylene blue. Polychrome indicators have been used for demonstrating neutral fats in the undifferentiated endodermis and aerial endodermis and for demonstrating free fatty acids in the differentiated endodermis in subaerial and etiolated stems (Priestley, 1926; Van Fleet, 1950). In the use of Nile blue sulphate, the blue salt of the red base forms blue salts with fatty acids, and the red dye particles dissolve in the neutral fats. Polychromed methylene blue gave a blue reaction with fatty acids in etiolated and subaerial tissues; under normal aerial conditions the same tissues stained lavender, indicating neutral fats.

MYROSULFATASE. The enzyme "myrosin" was regarded for a long time as a glucosidase that hydrolized sinigrin, the glucoside of mustard oil, and alliin, the glucoside of garlic. Neuberg and

Wagner (1926) have shown, however, that " myrosin " or " myrosinase " is a mixture of thioglucosidase and myrosulfatase. Myrosulfatase in the Cruciferae hydrolyzes the ethereal sulfate radicle of potassium myronate (the glucoside sinigrin) to release potassium bisulphate.

In the histochemical detection of myrosulfatase, Peche (1913) placed sections of radish in a solution of potassium myronate in saturated barium chloride. A precipitate, identified as barium sulfate, appeared after a short time in certain cells; the reaction occurred only in the presence of potassium myronate and not through the action of barium chloride alone. The reaction was strongest in the bundle zone and in idioblasts of the bundle sheath in leaves and stems, confirming earlier observations (Guignard, 1890*b;* Schweidler, 1905, 1910; Spatzier, 1893) in which unreliable tests for oil release from mustard oil glucoside were used. The older tests, for oil globules released from the glucosides, were not reliable because of the high amount of fatty oil already present in bundle sheath cells, as pointed out by Molisch (1923).

PHOSPHATASES. The histochemical technique for detecting alkaline phosphatases in animal tissues was developed independently by Gomori (1939) and Takamatsu (1939). In the method developed by Gomori for the detection of alkaline phosphatase, sections are incubated in a buffered glycero-phosphate substrate in the presence of calcium ions; phosphate freed by the phosphatase is precipitated at sites of enzyme activity as calcium phosphate, and a calcium stain is applied to the sections. In the technique for acid phosphatase developed by Gomori (1941), the incubation is carried out in the presence of lead ions, and the lead phosphate precipitate is converted to the black sulfide with ammonium sulfide. This method was first applied to a study of phosphatase localization in plant tissues by Hilbe and Marron (1941); they found localization of phosphatase in the actively growing parts of the embryo in germinating wheat.

The disadvantage of the Gomori method, according to Yin (1945), is interference by calcium and other insoluble salts present in the tissue. To avoid this difficulty, Yin used a method, applied earlier to animal tissue by Menten, Junge and Green (1943), in which sections were incubated with a calcium salt of naphthol phosphate and alpha-naphtholamine diazotized with sodium nitrite. The

phosphatase freed the naphthol as an insoluble purple-red azo dye. The diazotized amine reacted with lignified tissues to give an orange-red color which was, however, easily distinguished from the purple-red naphthol of the phosphatase reaction. Yin found the highest phosphatase activity in meristems, chlorenchyma, phloem and the endodermis and its homologue the bundle sheath. Glick (1949*b*, page 79) has pointed out that preformed phosphates may be removed with citrate buffer; hence this method offers no particular advantage over the standard Gomori method.

The Gomori method of converting liberated phosphate to lead phosphate followed by change of the lead phosphate to lead sulfide was adapted by Glick and Fischer (1945*a*, 1945*b*, 1946) to a study of phosphatases in wheat grains and germinating wheat. To conserve the substrates (adenosine triphosphate, thiamine pyrophosphate and glycerophosphate), a hanging drop technique was developed which employed one drop of substrate for each section of material. Control sections were used to indicate the pattern of free phosphate. Phosphatase was found to be higher in the cortex than in the stele of the root. Strong reactions were observed in the aleurone and scutellum, particularly in the bundles and bundle zone of the scutellum. The strongest reactions in the embryos were observed in the vascular bundles. Moog and Steinbach (1946) are of the opinion that the adenosine triphosphatase demonstrated by Glick and Fischer (1945*a*) is in reality a phosphomonoesterase, capable of hydrolyzing all three phosphate groups of adenosine triphosphate, and that adenosine triphosphatase is destroyed by the histological methods used. Glick (1949*a*) suggests that separation of alkaline phosphatases and adenosine triphosphatase may be pursued by observing differences in their localization or in their properties in sections.

Localization of acid phosphatase in germinating corn was studied by Kugler and Bennett (1947). The method of Gomori was used and the authors included a study of the distribution of preformed phosphate, employing Sumner's (1944) colorimetric method for phosphorus. The "axis" of the embryo, the scutellum and the aleurone gave strong positive reactions for phosphatase.

Wilson (1949) made a comprehensive and interesting correlative study of acid phosphatase patterns in several stages of the differentiation and development of cucurbit fruits. She used methods

of Gomori and Glick for the detection of phosphate liberated from glycerophosphate and prepared control sections for free phosphate of all stages studied. Differences in tissue distribution of phosphatase were more clearly defined in maturing fruits than in the very young ovaries. In young ovaries phosphatase action was relatively greater in vascular tissue, the placenta and the megasporangium. At the flowering stage the embryo sac, the surrounding nucellar cells and the extension of the nucellus into the micropyle were highly active. In later stages of fruit maturation, phosphatase was localized in the maturing ovules and their tissues of attachment. Localization of phosphatase was closely associated with differentiation and maturation and would appear to be significant in carbohydrate transformations taking place at critical stages in morphogenesis.

OTHER ESTERASES. Recent work on other esterases, acetylesterase in citrus and pectase in tobacco, is of interest. Holden (1946) found by extraction methods and determination of the amount of methanol liberated from pectin that the esterase pectase, which hydrolyzes methyl groups from pectin, is present in largest amount in the vascular tissue of the tobacco leaf, whereas the sap of the tobacco leaf contains only small amounts. Citrus acetylesterase, which hydrolyzes esters of acetic acid and has not been previously reported in plants, has been shown by extraction of portions of citrus fruit to be predominantly in the flavedo of the fruit with decreasing amounts toward the center of the fruit (Jansen, Jang and MacDonnell, 1947).

PHOSPHORYLASES

Phosphorylase in plant tissues synthesizes starch; it brings about the reversible reaction starch + phosphate $\leftrightarrows$ glucose-1-phosphate. It was discovered in plants by Hanes (1940) who obtained glucose-1-phosphate when phosphorylase extracted from peas was digested with soluble starch and phosphate. Synthesis of starch from glucose-1-phosphate by phosphorylase from plants may readily be demonstrated in the tissue by iodine tests for starch.

Yin and Sun (1947) and Yin (1948) were the first to devise a histochemical method for the detection of phosphorylase in plant tissue. Sections from soy beans were incubated in glucose-1-phosphate with a buffer and toluol; starch grains were observed after

30 to 60 minutes when the sections were stained with iodine in potassium iodide. That starch formation was a result of phosphorylase action and not of phosphatase or amylase was demonstrated by the following: no starch was found until after 12 hours of incubation when glucose was used as the substrate in lieu of glucose-1-phosphate; no effect was produced by sodium fluoride, a phosphatase inhibitor; boiled sections gave no reaction. Phosphorylase was found by Yin and Sun to be strongly localized in the rootcap, less strongly in meristems and still less in young leaves, stem tips and hypocotyl, with least in cotyledons.

Phosphorylase in leaves was found to be localized in the chloroplasts (Yin, 1948). It is of interest that aldolase, which catalyzes the reversible reaction fructose diphosphate $\rightleftarrows$ 2 triose phosphates, and participates in phosphorylytic processes, has been found by Tewfik and Stumpf (1949) in the cytoplasm and not in the chloroplasts in fractionation studies of leaf material.

Yin and Sun (1949), using glucose-1-phosphate as substrate and subsequent iodine staining of starch formed in germinating soy beans, found a general coincidence of the sites of phosphorylase activity with those of normal starch synthesis. Cytological observation revealed that phosphorylase was localized in the plastids.

The distribution of phosphorylase was studied by Sun and Yin (1948) in a number of vascular plants, and the highest activity was found in the embryo and meristems; they classified the intensity of activity for all of the major tissues for several plants. Yin and Sun (1947) demonstrated earlier that the abundance and size of the starch grains formed per cell may be used to indicate the intensity of phosphorylase activity.

By introducing modifications in the method of Yin and Sun, some interesting observations were obtained by Dyar (1950) on starch synthesis in pea root tips. She found that phosphorylase activity was strongest in root cap cells, high in the meristem and procambium, lower in the cortex. Phosphorylase activity was checked by the previously unclassified starch synthesis inhibitor from onion tissue found by Eyster (1949). Acid phosphatase distribution within root cap cells, determined by a lead sulfide precipitate method, was not like that of phosphorylase, and the distribution of acid phosphatase did not extend to elongating cells of the stele that contained phosphorylase. Dyar found phosphorylase in the

nuclei of root tip cells; that this was not an artifact was carefully checked because Yin and Sun (1949) reported no evident phosphorylase activity in the nuclei of root tip cells of soy beans.

PROTEOLYTIC ENZYMES

Histochemical determination of the enzymes that catalyze the hydrolysis of proteins has been studied by microtitration of the free amino groups formed. The most satisfactory methods used for estimation of peptidases and proteinases were developed by Linderstrøm-Lang and Holter (1931, 1932), and Engel and Heins (1947).

In the microtitration method developed for these studies, the tissue or tissue extract was added with glass needle or micropipette to a reaction tube, the substrate was added with a micropipette, and a glass bead filled with iron powder was used with an electromagnet as a stirrer. At the end of a given time period the reaction was stopped with alcoholic hydrochloric acid, and with a micro-burette designed for the purpose the quantity of amino groups released was determined by titration with hydrochloric acid.

Linderstrøm-Lang and Holter (1932, 1934) studied peptidase in root tips and cotyledons of malt grain and the proteinase in the glandular droplets of *Drosera* flowers. In the work on proteinases of the glandular hairs of *Drosera rotundifolia* (see Holter and Linderstrøm-Lang, 1933), edestin was used as the substrate, tissue samples were stirred in a reaction vessel and the result titrated with hydrochloric acid.

The enzymes of the glandular hairs were studied by collecting the secretion from the hairs on filter paper and extracting from the paper with glycerin; extracts were also prepared from the leaf blade and from the excised glandular hairs. It was found that the proteinase activity of the excretion collected from the glandular hairs exceeded that of the leaf tissue and of the excised glandular hairs. Two proteinases were detected: a secretion enzyme (pH optimum 3.2) from the glandular hairs and an endoenzyme (pH optimum 4.3) present in the leaf tissues.

The peptidase distribution in barley roots was observed in the intact roots or in longitudinal sections of the roots. The sections, cut at 200 microns, were obtained from roots placed in a low melting point paraffin without using the common dehydration/infiltration method. The sections or intact roots were placed in a reaction

vessel with alanylglycin and leucylglycin as substrates. Hydrolysis of these substrates was highest in the first few mm. of the root; it was correlated with the region of elongation and not the meristem. In seedling leaves, where the same methods were used, the peptidase activity was constant from the tip to the base of the leaves.

The peptidase distribution in the coleoptile of *Avena* at different stages in growth was studied by Avery and Linderstrøm-Lang (1940) in relation to the unit weight and the number of cells. The tissue was sectioned in paraffin at 125 or 250 microns and placed in glycerin in a reaction vessel, and the substrate alanylglycine (0.2 M) was added with a semi-automatic pipette. Incubation was carried out at 40° C four hours for splitting of the dipeptide; the reaction was stopped with 0.05 *N* HCl in 95 per cent alcohol. Cleavage of the substrate was carried out by microtitration with hydrochloric acid with naphthyl red as the indicator.

The peptidase activity, expressed in arbitrary peptidase units, decreased on a segment basis in older coleoptiles; peptidase activity was greater at the tip for any coleoptile four mm. or more in length. Correlations were found between the gradients in structure, auxin and peptidase activity.

In a study of enzyme distribution in resting cereal grains, Engel and Heins (1947) worked out the quantitative distribution of proteinase and dipeptidase in wheat grains. The sampling methods and histological techniques used were developed earlier by Linderstrøm-Lang and Engel (1937). The proteinase was determined by titration of free amino acids formed by the enzyme with edestine as the substrate. The dipeptidase activity was determined by titration of free amino acids released by the enzyme from dl-alanylglycine. The aleurone of wheat, rye and barley were rich in proteinase and dipeptidase, but the endosperm contained small or negligible amounts of these enzymes. Moderate amounts of proteinase were found in the embryo; the amount was too low to estimate the quantity in parts of the embryo. The dipeptidase activity was high in the entire embryo, especially in the radicle and epithelium of the scutellum. Previously only Pett (1935), using the methods of Linderstrøm-Lang, had done any work on the quantitative distribution of proteinase and dipeptidase in wheat grains. Pett separated the grain into five parts (hull, endosperm, scutellum,

cotyledon and radicle) and found the greatest change in the scutellum; he emphasized the scutellum as the site of enzyme formation and function. Pett did not separate the aleurone from the subaleurone layers; in the work by Engel and Heins this separation was made by appropriate sectioning, and they found the aleurone and not the scutellum to be rich in proteinase and dipeptidase.

Engel and Bretschneider (1947), in further studies on resting cereals, found no relation between the number of mitochondria and the amount of proteinase in various tissues of wheat grain. Horning (1933) had concluded earlier, on the basis of their reaction with azine dyes, that mitochondria contain proteolytic enzymes.

AMIDASES

UREASE. Urease is the only amidase that has been localized in plant tissue. It is present in jack beans, soy beans and various other beans. Its exact function is not known, but it probably hydrolyzes urea and prevents its accumulation. Glick (1949*b*) outlines a method, developed by Sen (1930), for localization of urease in the jack bean. The carbon dioxide formed from urea is converted to calcium carbonate or cobalt carbonate in the locus of the enzyme action by calcium or cobalt ions in the substrate medium. The calcium carbonate is converted to the visible silver carbonate and then to black metallic silver by reduction. The cobalt carbonate is converted to a visible precipitate of cobalt sulfide, and, according to Glick (1949*a*), the latter method is to be preferred.

Granick (1937*a*, 1937*b*) used the change in hydrion concentration as an index for a qualitative and quantitative study of the distribution of urease throughout the life history of *Canavalia ensiformis*, the jack bean. Increase in alkalinity, as a result of release of ammonia, was revealed by pH indicators or by the formation of a "lake". Urease was synthesized in the primary meristem and was not found in the cambium, phloem or xylem. Urease activity of the parenchymal cell was correlated with the physiological age of the cell. Granick (1938) found a similar distribution of urease in soy beans by examining microtome sections treated with the histological reagent he developed (aqueous haematoxylin, urea and nickel chloride). Formation of a non-diffusible nickel hydroxide-haematoxylin lake sharply localizes the distribution of this enzyme.

CONCLUSIONS

The significance of the localization of enzymes in some tissues is not apparent, but it is clear that certain tissues respond to qualitative and quantitative tests for enzymes, whereas others give very low or negligible reactions for the same enzymes. Several enzymes may be demonstrated by qualitative tests to be present in the differentiating cells of the parenchymal/vascular boundary. The sheathing cells of the border parenchyma, starch sheath and endodermis give strong reactions for several enzymes (polyphenoloxidase, peroxidase, myrosulfatase, esterases, carbohydrases, etc.). The periderm, epidermis and hypodermis, the boundary tissues between the environ and the body of the plant, also give reactions for more enzymes than adjacent tissues. The aleurone and subaleurone as peripheral and boundary tissues of the endosperm show by quantitative tests higher levels of enzyme action and more kinds of enzymes than other tissues of the seed. The reason for this localization of enzymes in the tissues that lie between two extremes or occupy a peripheral or surface position has not been elucidated. The protein content and enzyme reactivity of these cells that occupy a differential or interface position are apparently greater than for other tissues.

The phloem reacts readily for peroxidase in qualitative tests, and the presence of peroxidase, or leptomin as it was called by early botanists, is one of the histochemical characteristics of this tissue. Other enzymes, in particular the dehydrogenases, are also present in the phloem, but the dehydrogenases are more readily detected in other tissues.

The dehydrogenases are revealed by qualitative tests to be detectible in and perhaps characteristic of the meristems. The proteolytic enzymes and urease have been found by qualitative and quantitative tests to be higher in the growing region. Phosphorylases and phosphatases have also been found primarily in the meristems of the plant. A survey of all the enzymes in meristems has not been made, but it is reasonable to assume that many other enzymes are functional and present in high amount.

It is necessary to reiterate that, while the ultimate goal of the histochemist should be to study quantitative catalyst relationships, the topographic and qualitative aspects of demonstrating the histochemical localization of enzymes for many tissues in vascular plants

have yet to be realized. The presence or absence of a given enzyme in a tissue, correlated with some phase of differentiation of the tissue, is of value in categorizing the patterns and fields in differentiation, but even these comparatively simple studies have not been made. Of the 100 or more enzymes known to be in plants, less than 20 have been qualitatively localized in vascular plants. A statement of principle should be offered as a stimulus for the physiological anatomist, the histologist, the student of morphogenesis for studying enzyme distribution in the plant. Enzymes are the catalysts in the plant; their distribution and function are the actions and processes of histogenesis.

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